University of Alberta

The Regulation of Protein Phosphatase-1 by Reversible Protein Phosphorylation and Marine Toxins

by

John Francis Dawson ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

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John Dawson

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May 11, 1998

"Let us begin by committing ourselves to the truth
-- to see it as it is, and tell it like it is --
to find the truth,
to seek the truth,
and to live the truth."

Richard M. Nixon

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Regulation of Protein Phosphatase-1 by Reversible Protein Phosphorylation and Marine Toxins submitted by John Francis Dawson in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Protein phosphatase-1 (PP-1) is one of only four major protein serine/threonine phosphatases responsible for maintaining phosphorylation homeostasis in eukaryotic cells. PP-1 regulation consists of a complex combination of several mechanisms, including association of regulatory subunits and reversible protein phosphorylation. PP-1 is also regulated by marine toxins including okadaic acid, microcystins and nodularins.

A novel method for detection and measurement of protein phosphorylation employing capillary electrophoresis was developed and provided evidence supporting the hypothesis that glycogen synthase, a physiological PP-1 substrate, is phosphorylated in a hierarchical fashion. This technique was also applied to novel substrates and systems throughout the thesis.

Signal transduction in the marine dinoflagellate *Prorocentrum lima*, a primary producer of okadaic acid, was investigated. It was found that *P. lima* possesses signal transduction molecules that are similar to those in eukaryotes, suggesting that dinoflagellates are not mesokaryotes as had been previously proposed. This work represents the first biochemical characterization of tyrosine protein kinases and phosphatases in marine dinoflagellates.

The hypothesis that PP-1 among several lower eukaryotes may be regulated by direct phosphorylation of its catalytic subunit by PKA was tested in the fission yeast *Schizosaccharomyces pombe* where an inhibitor-resistant form of PP-1 was identified . This work provides the first clear evidence that different isoforms of PP-1 might play different roles in various physiological mechanisms. *S. pombe* PP-1 was not phosphorylated by PKA. Higher order constraints in the region surrounding the consensus sequence may prevent its phosphorylation.

Inhibition of PP-1 by marine toxins was investigated employing a mutagenesis strategy that targeted PP-1 sidechains that interact with the toxins. These studies support

the hypothesis that marine toxins bind to PP-1 within the same region through subtly different interactions.

Since PP-2B (calcineurin, a calcium/calmodulin-regulated protein phosphatase) possesses similar protein folding to PP-1 surrounding the toxin binding site of PP-1, an explanation for resistance of PP-2B to the marine toxins was investigated. This work culminates in a hypothesis that explains the structural rationale for PP-2B resistance to marine toxins.

To Mom.

Thank you will never be enough.

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Finally, I am thankful to my Lord for three things. First, for the blessing of the Rocky Mountains to remind me of the power of nature, both overwhelmingly beautiful and horribly dangerous. Second, I am thankful for the gift of music in my life to express the soaring joy and desperate sorrow in my heart. And third, I praise God for His showering of love down upon me, in the form of the many people in my life and in my fiancée Amanda Lawrence who has demonstrated a depth of faith and grace that knows no bounds.

I look forward to the future with the sorrow of times gone by and the anticipation of newness to come.

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List of Abbreviations

Proteins

ABI1	isoform of protein phosphatase-2C in *Arabidopsis*
bimG11	isoform of protein phosphatase-1 in *A. nidulans*
BSA	bovine serum albumin
CK-II	casein kinase-II
CREB	cAMP-response element binding protein
DARPP-32	dopamine and cAMP-regulated phosphoprotein-32; specific inhibitor of protein phosphatase-1
dis2	isoform of protein phosphatase-1 in *S. pombe*
G_L subunit	glycogen-binding subunit of PP-1G from liver
G_M subunit	glycogen-binding subunit of PP-1G from skeletal muscle
GS	glycogen synthase
GSK-3	glycogen synthase kinase-3
I-1	inhibitor-1; specific inhibitor of protein phosphatase-1
I-2	inhibitor-2; specific inhibitor of protein phosphatase-1
ISPK	insulin-stimulated protein kinase
KAPP	isoform of protein phosphatase-2C in *Arabidopsis*
KBPAP	kidney bean purple acid phosphatase
L5	ribosomal protein; binds protein phosphatase-1
M subunit	myosin-binding subunit of PP-1M
MAP kinase	mitogen-activated protein kinase
MBP	myelin basic protein
NCp7	HIV-1-encoded protein; activator of protein phosphatase-2A
NFAT	nuclear factor transcription
NIPP	nuclear inhibitor of protein phosphatase-1
p53BP2	p53 binding protein-2
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKI	specific inhibitor of cAMP-dependent protein kinase
PLPKA	cAMP-dependent protein kinase from *Prorocentrum lima*
PLPP-1	protein phosphatase-1 from *Prorocentrum lima*
PLPTP	protein tyrosine phosphatase from *Prorocentrum lima*
PMPTP	protein tyrosine phosphatase from *Prorocentrum micans*
PNPTP	protein tyrosine phosphatase from *Prorocentrum minimum*
PP-1	protein phosphatase-1
PP-1c	catalytic subunit of protein phosphatase-1
PP-1G	complex of PP-1c and G subunit
PP-1M	complex of PP-1c and M subunit
PP-1N	complex of PP-1c and NIPP-1 or 2
PP-1R	complex of PP-1c and RIPP-1
PP-2A	protein phosphatase-2A
PP-2B	protein phosphatase-2B, calcineurin
PP-2C	protein phosphatase-2C
PP-r	inhibitor resistant protein phosphatase from *S. pombe*
PPIP46	protein phosphatase inhibitor protein, 46 amino acid fragment; active fragment of inhibitor-1
pRB	retinoblastoma protein
PSF	splicing factor; binds protein phosphatase-1
PTG	glycogen-binding subunit; interacts with protein phosphatase-1
PTK	protein tyrosine kinase

R5	glycogen-binding subunit; interacts with protein phosphatase-1
RIPP-1	ribosomal inhibitor of protein phosphatase-1
RLK5	protein serine/threonine kinase cell-surface receptor in *Arabidopsis*
sds21	isoform of protein phosphatase-1 in *S. pombe*
vpr	HIV-encoded protein; activator of protein phosphatase-2A

Standard Amino Acids

Name	3-Letter Code	1-Letter Code
Nonpolar		
Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Proline	Pro	P
Phenylalanine	Phe	F
Tryptophan	Trp	W
Uncharged Polar		
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y
Asparagine	Asn	N
Glutamine	Gln	Q
Cysteine	Cys	C
Charged		
Lysine	Lys	K
Arginine	Arg	R
Histidine	His	H
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E

Special Amino Acids

pSer	phosphoserine
pThr	phosphothreonine
pTyr	phosphotyrosine
Adda	[2S, 3S, 8S, 9S]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Masp	methyl aspartic acid
Mdha	N-methyldehydroalanine
Mdhb	N-methyldehydrobutyrine

Biochemical and GeneticTerms

CRE	cAMP-responsive element
dis2$^+$	gene in *S. pombe* encoding the dis2 isoform of protein phosphatase-1
G phase	phase of the eukaryotic cell cycle in which growth of the cell occurs

HA	hemagluttinin epitope
IC_{50}	concentration of inhibitor causing 50% inhibition of an enzyme activity
LD_{50}	50% of the lethal concentration of a toxin in a given organism
M phase	phase of the eukaryotic cell cycle in which mitosis occurs
PPP	phosphoprotein phosphatase gene family, including PP-1, PP-2A and PP-2B
PPM	phosphoprotein phosphatase, magnesium-dependent gene family; includes PP-2C
S phase	phase of the eukaryotic cell cycle in which DNA synthesis occurs
sds21$^+$	gene in *S. pombe* encoding the sds21 isoform of protein phosphatase-1

Reagents and other Compounds

ATP	adenosine 5'-triphosphate
cAMP	cyclic- 3',5'- adenosine monophosphate
CBQCA	3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
IPTG	isopropyl-β-D-thiogalactoside
PMSF	phenylmethylsulfonyl fluoride
*p*NPP	para-nitrophenolphosphate
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate

Technical Terms

EOF	electroosmotic flow; influences capillary electrophoresis
FPLC	fast-performance liquid chromatography
HPLC	high-performance liquid chromatography
LIF	laser-induced fluorescence
PCR	polymerase chain reaction
RPLC	reverse-phase liquid chromatography
SDS-PAGE	polyacrylamide gel electrophoresis including SDS
UV	ultraviolet

Units and Constants

Å	Ångstroms (10^{-10} meters)
°C	degrees Celsius
Ci	Curies
cm	centimeters (10^{-2} meters)
cpm	counts per minute
dpm	degradations per minute
g	acceleration due to gravity

kDa	kiloDaltons
K_M	Michaelis-Menton constant
kV	kilovolts
M	molar (moles per liter)
min	minutes
mL	milliliters (10^{-3} liters)
mm	millimeters (10^{-3} meters)
mM	millimolar (10^{-3} moles per liter)
mU	milliunits (10^{-3} units)
nm	nanometers (10^{-9} meters)
nM	nanomolar (10^{-9} molar)
pH	-log of the concentration of H^+ in solution
pmol	picomoles (10^{-12} moles)
r_{av}	average radius
U	unit
v/v	volume per volume
w/v	weight per volume
µCi	microCuries (10^{-6} Curies)
µg	micrograms (10^{-6} grams)
µl	micoliters (10^{-6} liters)
µm	micrometers (10^{-6} meters)
µM	micromolar (10^{-6} molar)

Chapter 1

Introduction

1.1 ■ SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

Living cells respond to stimuli. These stimuli (or signals) come from inside or outside the cell. Life is dependent on cells correctly receiving and appropriately responding to signals: a cell that cannot respond to an intracellular requirement for nutrients quickly dies of starvation. Likewise, a cell that cannot remove itself from hostile environments meets a lethal fate.

Understanding how cells receive and respond to signals on the biochemical level is a key objective of those studying signal transduction. As cells have evolved from prokaryote to unicellular eukaryote to multicellular eukaryote, signal transduction mechanisms have become more complex to accommodate the increasing challenge of coordinating growth and development and maintaining metabolic balance.

A principal molecular mechanism by which cells respond to signals is reversible protein phosphorylation. The reversibility of this process is governed by the concerted and antagonistic actions of protein kinases (PKs) and protein phosphatases (PPases) (Fig. 1.1) [1]. Throughout evolution, two main strategies for reversible protein phosphorylation have emerged. Among prokaryotes, histidine amino acid residues are subject to reversible protein phosphorylation, while among eukaryotes, serine, threonine, or tyrosine residues are the primary targets.

This thesis will focus on reversible protein phosphorylation among eukaryotic cells. Eukaryotic protein kinases catalyze the transfer of the γ-phosphoryl group from ATP to serine, threonine, or tyrosine residues, thereby effecting a change in the conformation of target enzymes and altering their catalytic efficiency or function. This diverse family of enzymes ranges from large hormone receptors that span the plasma membrane to cell cycle-control protein kinases [2]. There are two major classes of protein kinases: those that phosphorylate specifically on serine or threonine residues [3]

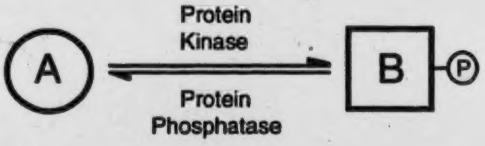


Fig. 1.1 Reversible protein phosphorylation. The reversibility of protein phosphorylation is governed by the antagonistic actions of protein kinases and protein phosphatases. Although only one phosphorylation is shown, the majority of phosphoproteins are phosphorylated on multiple sites. As illustrated above, protein A is phosphorylated into form B. Phosphorylation of enzymes often causes changes in protein conformation that translate into differences in catalytic efficiency.

and those that show specificity for tyrosine residues [4]. There also exists a third class of protein kinases, referred to as the dual-specificity protein kinases, which phosphorylate both tyrosine residues and serine and threonine residues. The fundamental influence of protein kinases on cellular homeostasis is demonstrated by the many oncogenic protein kinases that disrupt normal signaling pathways [5,6].

Protein Serine/Threonine Kinases

An excellent example of a signal transduction pathway involving protein phosphorylation is the physiological response to adrenalin, which elicits a multitude of biochemical changes. When adrenalin is bound by its cognate receptor on the surface of cells, adenylate cyclase is activated, resulting in the intracellular production of cyclic adenosine 3', 5'-monophosphate (cAMP). In this manner, the initial external hormone signal (the primary messenger) is transduced into an internal cAMP signal (the second messenger) (Fig. 1.2).

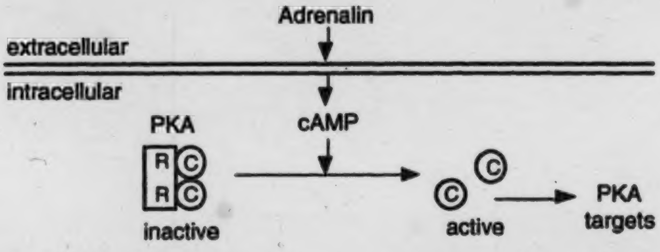


Fig. 1.2 Activation of cAMP-dependent protein kinase. Increases in intracellular cAMP in response to adrenalin cause the dissociation of inhibitory regulatory (R) subunits from inactive PKA complexes, releasing active catalytic (C) subunits which phosphorylate PKA target proteins.

cAMP-dependent protein kinase (PKA) exists in an inactive form as a tetramer consisting of two inhibitory regulatory (R) subunits and two catalytic (C) subunits [7-10]. Binding of cAMP to the R subunits of the tetramer causes conformational changes in the R subunits that result in the dissociation of two active C subunits and an $R_2(cAMP)_4$ dimer. The free C subunit then phosphorylates target proteins in the cytoplasm and nucleus, thereby regulating a number of cellular effects that are manifested as the physiological response to cAMP. Among these are the regulation of glycogen synthase and phosphorylase kinase activity to contribute to the upregulation of ATP production for use in the "fight or flight" response. In addition, PKA also alters the pattern of gene expression in the nucleus by regulating the function of transcription factors that bind to DNA sequences in the promoter regions of cAMP-inducible genes [11,12]. Thus, the pleiotropic effects of a single input signal can be effected through activation of a single protein kinase with multiple intracellular substrates.

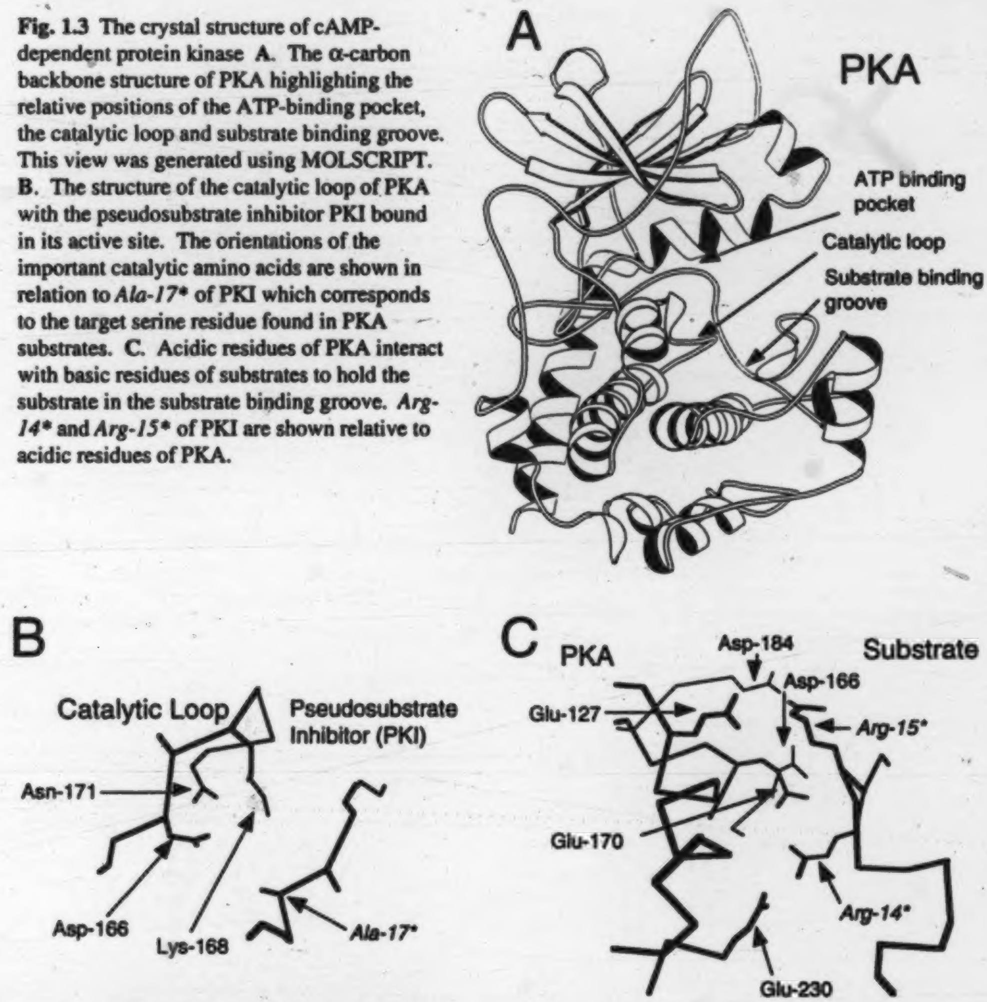
There are numerous physiological processes in cells that require regulation by phosphorylation. The diversity of these processes in humans suggests an equally numerous diversity of protein kinases encoded by our genome. Recent estimates place the number of distinct protein kinases encoded by the human genome at somewhere between 1000 and 2000 [1], a reflection of the important physiological processes governed by protein phosphorylation. The importance of protein kinases is also seen in the recently sequenced genome of the budding yeast *Saccharomyces cerevisiae*, where 2% of all genes are allocated to encoding 113 different protein kinases, of which only 60% have a known or hypothesized function [13].

Catalytic Mechanism of Protein Kinases

Recently, several protein serine/threonine kinase crystal structures have been determined [14-21]. The overall organization of these protein serine/threonine kinases consists of a bilobal structure in which ATP is bound in the cleft between the two lobes and the carboxy-terminal lobe contains the substrate-binding machinery (Fig. 1.3A). The smaller amino-terminal lobe binds Mg-ATP, positioning the γ-phosphate for transfer. The adenine base moiety of ATP is held in place by a hydrophobic pocket while a lysine residue coupled ionically to a glutamic acid residue interacts with the non-transferable α and β-phosphates. These sidechains are among several conserved residues that are characteristic of protein serine/threonine kinases and are represented in PKA by Lys-72 and Glu-91 [2].

Catalysis among protein kinases is believed to occur through a catalytic aspartic acid residue which partially deprotonates the hydroxyl group of the target serine or threonine residue, catalyzing nucleophilic attack by the hydroxyl oxygen on the γ-phosphate of ATP [10]. Inversion of the phosphorus configuration occurs during phosphotransfer, suggesting that the γ-phosphate is positioned in-line with the target hydroxyl group. This mechanism is facilitated by three residues invariant among protein kinases and present in PKA as Asp-166, Lys-168 and Asn-171 on the catalytic loop found between β-sheets 6 and 7 of the larger carboxy-terminal lobe (Fig. 1.3B). Lys-168 interacts with the γ-phosphate of ATP to correctly orient it for interaction with the catalytic Asp-166. The invariant Asn-171 stabilizes the overall positioning of this interaction by hydrogen-bonding with the catalytic Asp-166 and ligating the Mg^{2+} in the ATP-binding site.

Fig. 1.3 The crystal structure of cAMP-dependent protein kinase A. The α-carbon backbone structure of PKA highlighting the relative positions of the ATP-binding pocket, the catalytic loop and substrate binding groove. This view was generated using MOLSCRIPT. B. The structure of the catalytic loop of PKA with the pseudosubstrate inhibitor PKI bound in its active site. The orientations of the important catalytic amino acids are shown in relation to *Ala-17** of PKI which corresponds to the target serine residue found in PKA substrates. C. Acidic residues of PKA interact with basic residues of substrates to hold the substrate in the substrate binding groove. *Arg-14** and *Arg-15** of PKI are shown relative to acidic residues of PKA.


A
PKA
ATP binding pocket
Catalytic loop
Substrate binding groove
B
Catalytic Loop
Pseudosubstrate Inhibitor (PKI)
Asn-171
Asp-166
Lys-168
Ala-17*
C
PKA
Asp-184
Substrate
Asp-166
Glu-127
Arg-15*
Glu-170
Arg-14*
Glu-230

Protein Kinase Substrate Specificity

Substrate specificity among protein kinases is achieved through variability in the substrate binding groove of the protein kinase which extends across the face of the large carboxy-terminal lobe, running over the catalytic loop under the small amino-terminal lobe [22,23]. In the case of PKA, the most preferred phosphorylation consensus sequence is Arg-Arg-Xaa-Ser [24,25]. However, there is great diversity in this sequence among PKA substrates, with only about a third actually containing this sequence. The substrate binding groove of PKA contains acidic residues critical for interactions with the arginine sidechains of the preferred substrate (Fig. 1.3C). In addition to these interactions, PKA also possesses two hydrophobic pockets which contribute to high-affinity binding of substrates. Consequently, there is a considerable variability of possible substrate-enzyme interactions and different substrates may make some, but not all, of the available contacts. As a result, protein kinases recognize a group of related target structures rather than a strict consensus sequence. Variability in protein kinase recognition may have important implications for signal transduction, since higher-affinity substrates may be preferentially phosphorylated upon kinase activation, thereby influencing the timing of protein phosphorylation within the cell. The discovery that different consensus sequences play an important role in the specificity of protein kinases lead to the use of peptides containing these consensus sequences for the detection, purification, and characterization of various protein serine/threonine kinase activities (Table 1.1) [26,27].

Protein kinases are mediators of reversible protein phosphorylation, a process that is vital to signal transduction and thus the ability of cells to respond to stimuli. However, protein kinases are only half of the story. The reversible nature of protein phosphorylation is dependent on another equally important class of proteins to catalyze the removal of phosphate groups from phosphorylated residues: the protein phosphatases. Understanding the regulation of protein phosphatases is critical to our understanding of reversible protein phosphorylation and, ultimately, how cells adapt to changes in their environment. The work described in this thesis is focused on this goal.

Table 1.1 Common protein serine/threonine kinase phosphorylation consensus sequences. The most preferred consensus sequence for selected protein serine/threonine kinases are listed. Phosphorylation sites are indicated in bold. Phosphoserine residues are indicated by Ser(P). Variations of these sequences are commonly targets of their respective kinase, as discussed in the text.

Kinase	Phosphorylation Consensus Sequence
cAMP-dependent protein kinase	Arg-Arg-Xaa-**Ser**
glycogen synthase kinase-3	**Ser**-Xaa-Xaa-Xaa-Ser(P)
casein kinase-I	Ser(P)-Xaa-Xaa-**Ser**
casein kinase-II	**Ser**-Xaa-Xaa-Glu
cdc2 kinase	**Thr**-Pro-Xaa-Arg/Lys
protein kinase C	Arg/Lys-Xaa-Xaa-**Ser**-Xaa-Arg/Lys

1.2 ■ PROTEIN PHOSPHATASES

Eukaryotic protein phosphatases (PPases) catalyze the hydrolysis of phosphate groups from phosphoserine (pSer), phosphothreonine (pThr), or phosphotyrosine (pTyr) on their target substrates and are therefore essential to the reversible nature of protein phosphorylation [28,29].

Protein Serine/Threonine Phosphatases

Like the protein kinases, protein phosphatases are subdivided into two major classes: those that dephosphorylate pSer or pThr residues and those that remove phosphate from pTyr residues. In addition, there is also a small family of dual-specificity protein phosphatases that dephosphorylate both pTyr residues and pSer and pThr residues. Unlike the protein kinases, however, there are only four major protein serine/threonine phosphatases known, represented by two distinct gene families [29-31]. The most abundant eukaryotic protein serine/threonine phosphatases, PP-1, PP-2A, and PP-2B, belong to the phosphoprotein phosphatase family, termed the PPP gene family in accordance with the three-letter nomenclature established for the Human Genome Project [32], while PP-2C belongs to the PPM gene family, an acronym for "protein phosphatase, Mg^{2+}-dependent" [32].

The PPM gene family is a large and diverse group of Mg^{2+}-dependent protein serine/threonine phosphatases found in both eukaryotes and prokaryotes. Substrate specificity is often achieved through the fusion of protein recognition domains to the catalytic domain of PP-2C. For example, the KAPP protein in *Arabidopsis* encodes a PP-2C catalytic domain fused to a kinase interaction (KI) domain. This permits KAPP interaction with specific pSer residues of the receptor protein serine/threonine kinase RLK5 in a similar fashion to SH2-domain interaction with distinct pTyr residues on receptor protein tyrosine kinases (PTKs) [33].

One of the roles of PP-2C is to regulate responses to stress. Again in *Arabidopsis*, mutations in the ABI1 isoform of PP-2C drastically reduce a range of responses to abscisic acid, including reduced seed dormancy, excessive water loss, and abnormal root growth [34,35]. In budding yeast, PP-2C down-regulates a MAP kinase-related pathway responsible for osmotic and heat shock response [36].

The PPP Family

The PPP family consisting of PP-1, PP-2A and PP-2B possesses highly conserved catalytic domains, suggesting similar protein folding and catalytic mechanisms (Fig. 1.4) [37]. PP-1 and PP-2A function independent of divalent cations, while PP-2B (also known as calcineurin) is Ca^{2+}-dependent (Table 1.2) [38]. PP-1 and PP-2A account for most of the measurable protein serine/threonine phosphatase activity in cell extracts and possess broad and overlapping substrate specificity *in vitro*, but can be differentiated by their preference to dephosphorylate the β or α subunit of phosphorylase kinase, respectively, and the specific inhibition of PP-1 by a group of endogenous inhibitor proteins, including inhibitor-1 and inhibitor-2 [39], discussed in detail in section 1.4. The focus of the work outlined in this thesis is on PP-1 activity. However, I shall touch on some of the important regulatory roles of each member of the PPP family.

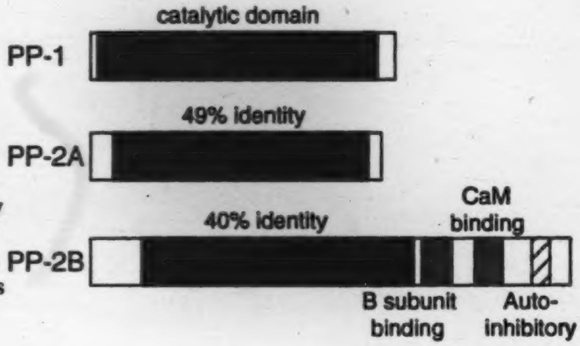


Fig. 1.4 Conservation of PPP family catalytic domains. PP-2A shares 49% identity with PP-1 in its catalytic domain, while PP-2B shares 40% identity in its catalytic domain. PP-2B also possesses a C-terminal tail which includes binding domains for the B subunit and calmodulin (shaded boxes) as well as the autoinhibitory domain (hatched box). Such a high degree of identity in the catalytic domain suggests that the PPP family of phosphatases possess similar protein folding and common catalytic mechanisms.

Protein Phosphatase-2B (Calcineurin)

The catalytic core of the PP-2B A-subunit shares 40% identity with PP-1. A carboxy-terminal α-helical extension on the PP-2B A-subunit catalytic core contains binding domains for the PP-2B B-subunit and calmodulin, with an autoinhibitory sequence located at the extreme carboxy-terminus. PP-2B is characterized by its dependence on Ca^{2+} binding to the B-subunit and calmodulin for maximal activity [40]. PP-2B regulates several signal transduction pathways having pleiotropic effects, but is most commonly associated with T-cell activation [41-43]. PP-2B dephosphorylates the cytosolic subunit of the NFAT transcription factor in T-cells presented with antigen [44], resulting in the translocation of NFAT to the nucleus and ultimately inducing the expression of the IL-2 gene [45], one of the early genes in T-cell activation. Inhibition of PP-2B by immunosuppressive drugs FK506 and cyclosporin bound to their cognate intracellular receptors suppresses T-cell activation [46-48]. In addition, PP-2B mediates PP-1 activity

Table 1.2 The biochemical characteristics of the PPP family of protein serine/threonine phosphatases. Endogenous inhibitor proteins inhibitor-1 (I-1) and inhibitor-2 (I-2) are discussed in section 1.4. Inhibition by the marine toxins okadaic acid, microcystin-LR and nodularin is discussed in section 1.6. The IC_{50} of nodularin for PP-2B was determined for this thesis.

	PP-1	PP-2A	PP-2B
Molecular mass (kDa)	37	36	61
Divalent cation requirement	none	none	Ca^{2+}
Phosphorylase kinase subunit preference	β	α	α
Inhibition by I-1 and I-2	yes	no	no
Inhibition by okadaic acid	yes (IC_{50} = 20 nM)	yes (IC_{50} = 0.2 nM)	v. weak (IC_{50} = 5000 nM)
Inhibition by microcystin-LR	yes (IC_{50} = 0.2 nM)	yes (IC_{50} = 0.2 nM)	v. weak (IC_{50} = 200 nM)
Inhibition by nodularin	yes (IC_{50} = 0.2 nM)	yes (IC_{50} = 0.2 nM)	v. weak (IC_{50} = 2000 nM)

by regulating the activity of the PP-1 endogenous inhibitor proteins, inhibitor-1 and DARPP-32, discussed in section 1.4. Briefly, dephosphorylation of inhibitor-1 and DARPP-32 by PP-2B causes the inhibitors to be ineffective, thereby allowing free PP-1 to be active in the cell.

Protein Phosphatase-2A

PP-2A shares 49% identity in its catalytic domain and broad and overlapping *in vitro* substrate specificity with PP-1. Like PP-2B, PP-2A is involved in regulating a host of varying physiological processes having pleiotropic effects [49]. PP-2A interacts with middle T antigen from polyoma viruses [50] and SV40 small T antigen [51], resulting in cell proliferation, suggesting a role for PP-2A in the regulation of cell growth. Inhibition of PP-2A *in vivo* by small T antigen impedes its dephosphorylation and downregulation of the MAP kinase cascade, resulting in constitutive receptor-mediated activation of these kinases and uncontrolled cell proliferation. Conversely, activation of PP-2A by the HIV-1 encoded protein complex NCp7-vpr leads to cell cycle arrest in G_2 by increased dephosphorylation and inhibition of the cdc25 protein tyrosine phosphatase [52,53], resulting in hyperphosphorylation and inhibition of cdc2 kinase, the key cell cycle-dependent protein kinase for the G_2-M transition, as discussed in section 1.3 (see Fig. 1.9).

1.3 ■ PROTEIN PHOSPHATASE-1

Protein phosphatase-1 is among the most conserved eukaryotic enzymes throughout evolution, displaying sequence conservation that is greater than that for tubulin or histone H2A, among the slowest evolving proteins known [54,55]. Three isoforms of mammalian PP-1 catalytic subunits exist, referred to as the α, β, and γ isoforms. These isoforms are differentiated primarily by their carboxy-terminal sequences, although subtle differences are also present at the amino-terminal (Table 1.3). Like the other protein serine/threonine phosphatases, PP-1 possesses a diversity of *in vivo* substrates, thereby influencing an equally diverse range of physiological processes [54,56,57]. Among these are: glycogen metabolism, calcium transport, lipid metabolism, muscle contraction, protein synthesis, gene expression, and cell proliferation. Detailing all of the physiological processes in which PP-1 is involved is beyond the scope of this chapter. However, I will briefly discuss three specific examples.

Glycogen metabolism

Two key enzymes govern the regulation of glycogen metabolism in eukaryotic cells. Glycogen synthase catalyzes the rate-limiting step in the synthesis of glycogen by transferring uridine diphosphate (UDP) glucose formed by UDP-glucose pyrophosphatase from glucose-1-phosphate and UTP to the C4 hydroxyl of one of glycogen's nonreducing termini to form an α (1-4) glycosidic bond, thereby extending the chain of glycosyl units in the glycogen molecule. Alternatively, phosphorylase kinase specifically phosphorylates Ser-14 of glycogen phosphorylase, thereby activating its ability to cleave glycosyl units from glycogen to form glucose-1-phosphate, a preliminary step in the process of ATP production through glycolysis [58]. Both glycogen synthase and phosphorylase kinase are phosphoproteins that are physiological substrates of PP-1. When presented with an excitatory stimulus, the body requires the activation of glycogenolysis for the production of ATP and the inhibition of glycogen synthase such that glucose levels in the blood can remain high as a contingency for producing ATP to

Table 1.3 Alignment of the primary sequences of the three human PP-1 isoforms. The three isoforms are distinguished mainly by their carboxy-termini where significant sequence differences exist. The numbers across the top correspond to the amino acid positions in PP-1α. Tyr-306 is believed to be the target of the v-src and v-abl protein tyrosine kinases, while Thr-320 of the C-terminal Thr-Pro-Pro-Arg motif is phosphorylated by cdc2 kinase and may play a role in the regulation of PP-1 activity during the cell cycle, as discussed in section 1.5.

	10	20	30	40	50	60
PP-1α	MSDSEKLNLD	SIIGRLLEVQ	GSRPGKNVQL	TENEIRGLCL	KSREIFLSQP	ILLELEAPLK
PP-1β	MADGE-LNVD	SLITRLLEVR	GCRCGKIVQM	TEAEVRGLCI	KSREIFLSQP	ILLELEAPLK
PP-1γ	MADLDKLNID	SIIQRLLEVR	GSKPGKNVQL	QENEIRGLCL	KSREIFLSQP	ILLELEAPLK
	70	80	90	100	110	120
PP-1α	ICGDIHGQYY	DLLRLFEYGG	FPPESNYLFL	GDYVDRGKQS	LETICLLLAY	KIKYPENFFL
PP-1β	ICGDIHGQYY	DLLRLFEYGG	FPPESNYLFL	GDYVDRGKQS	LETICLLLAY	KIKYPENFFL
PP-1γ	ICGDIHGQYY	DLLRLFEYGG	FPPESNYLFL	GDYVDRGKQS	LETICLLLAY	KIKYPENFFL
	130	140	150	160	170	180
PP-1α	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTDCFNCLP	IAAIVDEKIF	CCHGGLSPDL
PP-1β	LRGNHECASI	NRIYGFYDEC	KRRFNIKLWK	TFTDCFNCLP	IAAIVDEKIF	CCHGGLSPDL
PP-1γ	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTDCFNCLP	IAAIVDEKIF	CCHGGLSPDL
	190	200	210	220	230	240
PP-1α	QSMEQIRRIM	RPTDVPDQGL	LCDLLWSDPD	KDVQGWGEND	RGVSFTFGAE	VVAKFLHKHD
PP-1β	QSMEQIRRIM	RPTDVPDTGL	LCDLLWSDPD	KDVQGWGEND	RGVSFTFGAD	VVSKFLNRHD
PP-1γ	QSMEQIRRIM	RPTDVPDQGL	LCDLLWSDPD	KDVLGWGEND	RGVSFTFGAE	VVAKFLHKHD
	250	260	270	280	290	300
PP-1α	LDLICRAHQV	VEDGYEFFAK	RQLVTLFSAP	NYCGEFDNAG	AMMSVDETLM	CSFQILKPAD
PP-1β	LDLICRAHQV	VEDGYEFFAK	RQLVTLFSAP	NYCGEFDNAG	GMMSVDETLM	CSFQILKPSE
PP-1γ	LDLICRAHQV	VEDGYEFFAK	RQLVTLFSAP	NYCGEFDNAG	AMMSVDETLM	CSFQILKPAE
	310	320				
PP-1α	KNKGKYGQFS	GLNPGGRPIT	PPRNSA--K-AKK			
PP-1β	K-KAKY-QYG	GLN-SGRPVT	PPRTANPPK--KR			
PP-1γ	K-K-K-----	-PNAT-RPVT	PPR-GMITKQAKK			

respond to the stimulus. As described earlier, the release of adrenalin under these circumstances stimulates PKA activity, thereby phosphorylating and inhibiting glycogen synthase while phosphorylating and stimulating phosphorylase kinase (Fig. 1.5).

When blood glucose levels are high, such as after a meal, the body stores the glucose in the form of glycogen for later use. Therefore, glycogen synthase activity must be stimulated and phosphorylase kinase activity inhibited, a situation opposite to that described above for adrenalin signaling. Release of insulin by the pancreas in this case causes the stimulation of insulin-stimulated protein kinase (ISPK) in cells which phosphorylates and stimulates the glycogen-associated form of PP-1 [59]. The resulting highly active form of PP-1 dephosphorylates glycogen synthase and phosphorylase

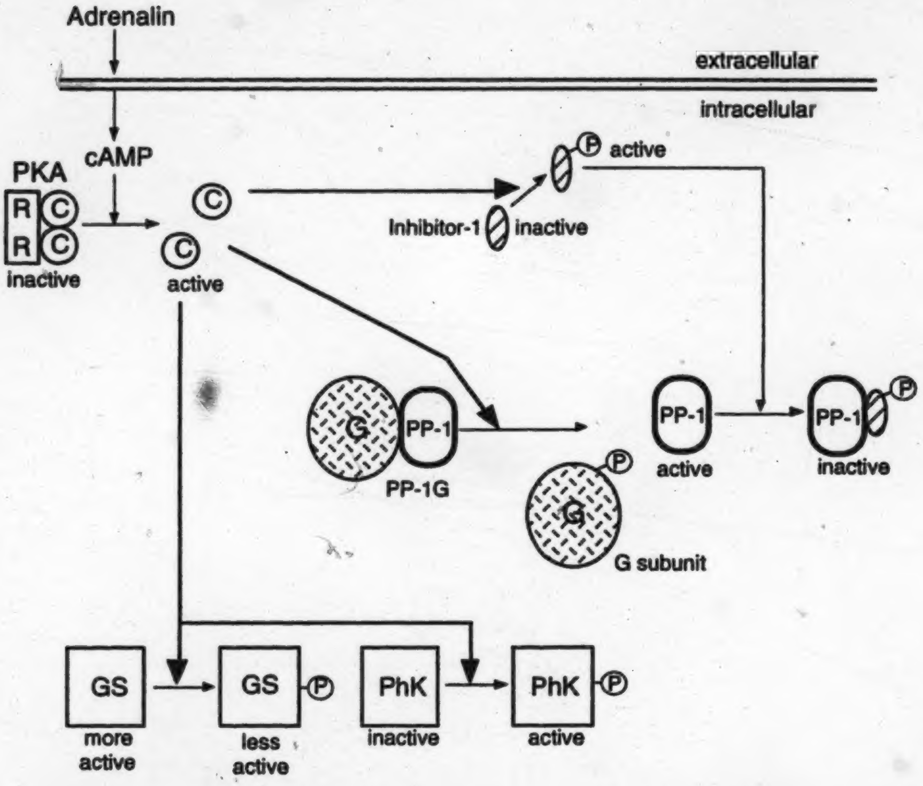

Adrenalin
extracellular
intracellular
cAMP
PKA
R
C
R
C
inactive
C
C
active
P
active
Inhibitor-1
inactive
G
PP-1
PP-1G
PP-1
active
PP-1
P
inactive
P
G
G subunit
GS
more active
GS
P
less active
PhK
inactive
PhK
P
active

Fig. 1.5 Regulation of key glycolytic enzymes in response to adrenalin. PKA phosphorylates a number of intracellular targets, including glycogen synthase (GS) and phosphorylase kinase (PhK). PKA downregulates PP-1 activity by dissociating PP-1 from glycogen particles and activating inhibitor-1 (I-1), which then binds and inhibits free PP-1.

kinase on the sites targeted by PKA, thereby reversing the effects of adrenalin signaling and resulting in glycogenesis (Fig. 1.6).

Hence, adrenalin and insulin have the opposite effect upon glycogen metabolism by stimulating the antagonistic activities of PKA and PP-1, respectively. However, the glycogen-associated form of PP-1 is still active without the stimulatory phosphorylation of ISPK and would be able to attenuate adrenalin signaling by dephosphorylating the accumulating products of PKA phosphorylation. In this situation, then, maximal response to adrenalin is not possible, due to the antagonistic effects of PP-1. However, we know that this is not the case. When adrenalin binds its cognate receptor, activated PKA phosphorylates the glycogen-associated form of PP-1 to dissociate PP-1 from the glycogen particles, thereby rendering it less active toward glycogen synthase and

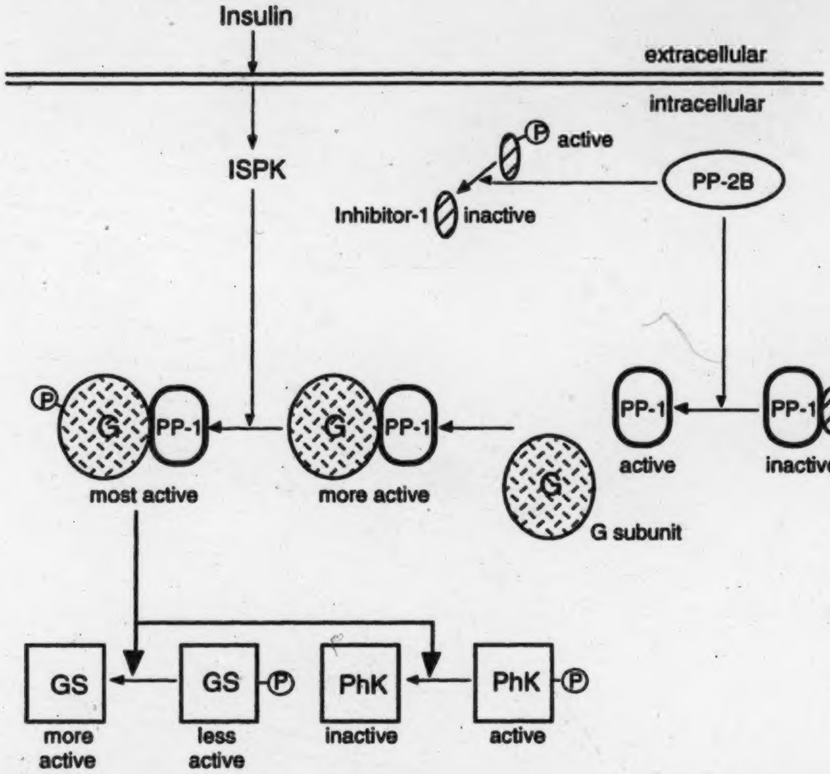

Insulin
extracellular
intracellular
ISPK
P
active
PP-2B
Inhibitor-1
inactive
P
G
PP-1
most active
G
PP-1
more active
G
G subunit
PP-1
active
PP-1
P
inactive
GS
more active
GS
P
less active
PhK
inactive
PhK
P
active

Fig. 1.6 Regulation of key glycolytic enzymes in response to insulin. PP-2B dephosphorylates and inactivates I-1 in the absence of PKA signaling. Free PP-1 then associates with glycogen particles through the G subunit. ISPK phosphorylates the glycogen-bound PP-1, increasing its activity toward glycogen synthase (GS) and phosphorylase kinase (PhK), resulting in increased glycogen synthesis and inhibition of glycogenolysis.

phosphorylase kinase (discussed in section 1.4). In addition, PKA phosphorylates and activates inhibitor-1 (I-1). Phosphorylated I-1 associates with free PP-1 and inhibits its activity during PKA signaling, permitting rapid and maximal stimulation of glycogenolysis and inhibition of glycogenesis.

Conversely, in the absence of PKA signaling, PP-2B dephosphorylates I-1, thereby inactivating it as an inhibitor of PP-1 and making PP-1 active in the cell. Free PP-1 is then able to associate with glycogen particles and be stimulated by insulin signaling to reverse the effects of PKA.

Gene expression

An increase in intracellular cAMP levels in eukaryotic cells results in changes in specific gene expression [11,60]. Most of these genes contain a cAMP-responsive element (CRE) possessing the consensus sequence TGACGTCA, although variations on this consensus exist [12]. This sequence is bound by a family of CRE-binding transcription factors, founded by the first CRE-binding protein to be cloned called CREB. The activity of CREB is regulated by reversible protein phosphorylation.

When intracellular levels of cAMP increase, phosphorylation of CREB on Ser-133 by activated PKA results in increased CREB activity and transcription of CRE-containing genes [61,62]. Once again, PP-1 opposes the action of PKA by dephosphorylating CREB and downregulating its transcriptional activator function. Again, PKA also phosphorylates I-1 to ensure that maximal response to the cAMP signal is achieved (Fig. 1.7).

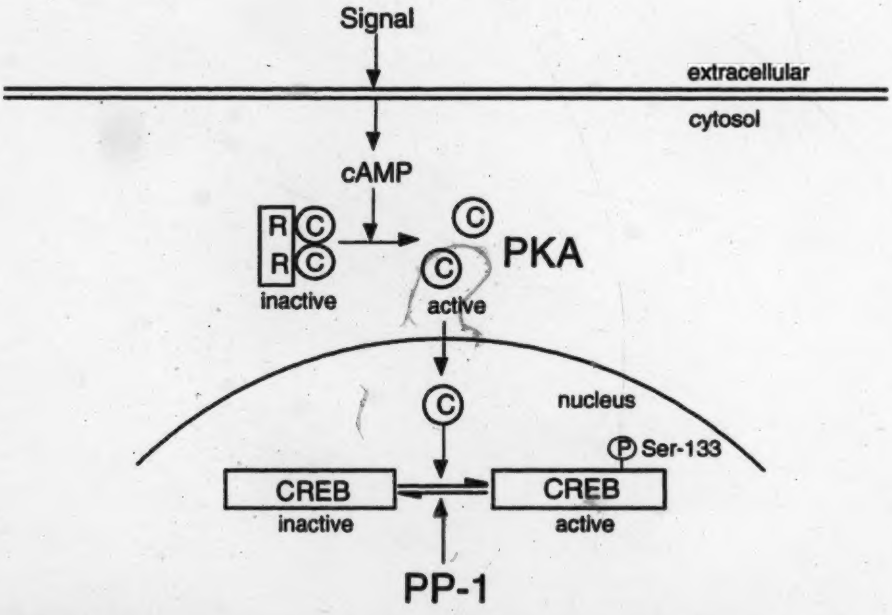


Fig. 1.7 Regulation of gene expression by protein phosphatase-1. PP-1 regulates the activity of the cAMP-response element binding protein (CREB) by dephosphorylating and downregulating its DNA binding activity, thereby counteracting the stimulatory activity of PKA on CREB.

Cell Cycle Control

The cell cycle is a series of events that result in the duplication and growth of eukaryotic cells. The cell cycle is typically divided into four phases consisting of DNA synthesis (S phase) and mitosis (M phase) separated by the G_1 and G_2 phases [63].

Several instances in the cell cycle serve as control points to ensure that each phase is complete before the beginning of the next. For example, DNA synthesis must be completed before mitosis can begin such that each daughter cell contains a full complement of genetic information. The signals that govern cell cycle control points are modulated by protein kinases and phosphatases. For example, progression into mitosis is regulated by a cyclin-dependent protein kinase referred to as $p34^{cdc2}$ in *S. pombe*. This kinase activity is high during mitosis due to association with cyclin B and low during interphase due to dissociation and degradation of cyclins at the end of mitosis (Fig. 1.8).

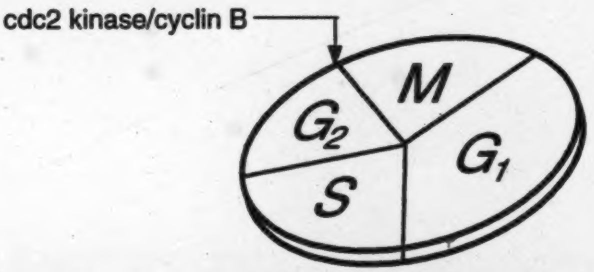


Fig. 1.8 The eukaryotic cell cycle is divided into distinct phases. The phase of DNA synthesis (S phase) and mitosis (M phase) are separated by cell growth (G) phases. Progession into M phase is regulated by the activity of the cdc2 kinase/cyclin B complex, which is itself regulated by reversible protein phosphorylation on tyrosine residues. The protein phosphatase that dephosphorylates and thereby activates cdc2 kinase is also the target of reversible protein phosphorylation and may be a substrate of PP-1.

Cdc2/cyclin B activity itself is regulated by reversible protein phosphorylation. The cdc2/cyclin B complex forms soon after S phase, but remains inactive during G_2 due to phosphorylation on Tyr-15 by the wee1 protein tyrosine kinase (PTK). Activation of kinase activity at the end of G_2 triggers mitosis and is catalyzed by the dephosphorylation of Tyr-15 by the cdc25 protein tyrosine phosphatase (PTPase). The activity of cdc25 is also regulated by reversible protein phosphorylation and oscillates during the cell cycle, being low during interphase and high in M phase. Increased activity in M phase correlates with increased phosphorylation on serine and threonine residues whereas dephosphorylation of cdc25 during interphase leads to hyperphosphorylation and inactivation of cdc2/cyclin B (Fig. 1.9).

The isolation of cell cycle mutants of *Aspergillus nidulans* and *S. pombe* has demonstrated the involvement of PP-1 in cell cycle control. The *A. nidulans* temperature-

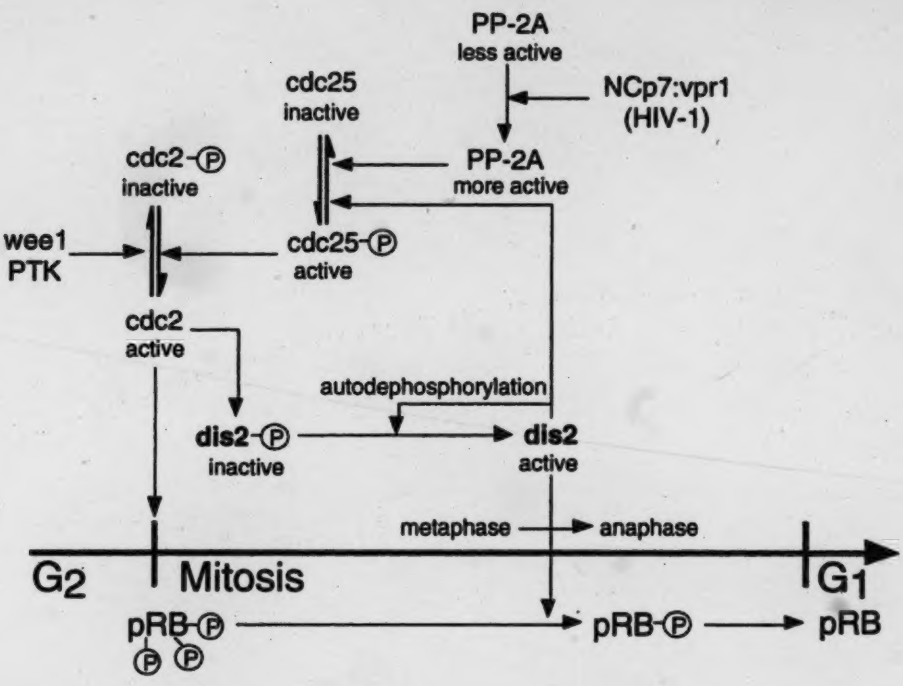

PP-2A
less active
NCp7:vpr1
(HIV-1)
cdc25
inactive
PP-2A
more active
cdc2-Ⓟ
inactive
wee1
PTK
cdc25-Ⓟ
active
cdc2
active
autodephosphorylation
dis2-Ⓟ
inactive
dis2
active
metaphase
anaphase
G2
Mitosis
G1
pRB-Ⓟ Ⓟ Ⓟ
pRB-Ⓟ
pRB

Fig. 1.9 Involvement of PP-1 in cell cycle control. Entry into mitosis is regulated by the activity of cdc2 kinase, which must be dephosphorylated by cdc25 phosphatase, itself regulated by phosphorylation, to be active. When dephosphorylated, cdc25 is inactive. Therefore, activating the phosphatases that dephosphorylate cdc25, including the PP-1 isoform dis2 in *S. pombe*, results in its inactivation. In turn, cdc2 becomes hyperphosphorylated and inactive, leading to delay in mitosis. Conversely, inhibiting PP-1 activity ultimately leads to premature entry into mitosis. PP-1 is also thought to control cell cycle progression by dephosphorylating the retinoblastoma protein. The hyperphosphorylated form of pRB that is required for entry into mitosis is therefore dephosphorylated upon exit from mitosis.

sensitive mutant bimG11 [64] and *S. pombe* cold-sensitive mutant dis2-11 [65,66] both exhibit blocks at anaphase of mitosis and fail to appropriately segregate daughter chromosomes. Both of these mutants encode PP-1 isoforms. In *Drosophila*, a mutation in one of the four PP-1 genes results in delayed progress through mitosis and defective mitotic spindle organization among other mitotic defects, which can be compensated for by re-introducing the wild type PP-1 gene into cells [67]. In addition, microinjection of PP-1-neutralizing antibodies into rat fibroblasts cause cell cycle arrest at metaphase, while microinjection of purified PP-1 accelerates exit from mitosis [68]. Moreover, a cell permeable inhibitor of PP-1 causes cell cycle arrest during the transition from metaphase to anaphase in porcine hepatocytes [69].

The distribution of PP-1 also fluctuates during the cell cycle, where PP-1 accumulates in the nucleus during G_2 and M phases and is associated with chromatin [68], suggesting a role for PP-1 dephosphorylation of nuclear or chromatin-associated proteins. The existence of such a role is supported by the observation of hyperphosphorylation of nuclear proteins in *A. nidulans* possessing a mutation of PP-1 [64].

In addition, the activity of PP-1 in *Xenopus* oocytes has been demonstrated to oscillate during the cell cycle, being high during S phase and low at the onset of M phase [70]. This is in contrast to results obtained in *S. pombe*, where PP-1 activity remained constant throughout the cell cycle [71]. However, it is likely that PP-1 activity toward the substrate used to measure PP-1 activity in *S. pombe* (phosphorylase) is not regulated during the cell cycle. Conversely, H1 histone was employed to assay *Xenopus* PP-1.

Clearly then, PP-1 plays a pivotal role in regulation of the cell cycle. Unfortunately, the specific substrates through which PP-1 exerts its regulatory function are not known. Potential substrates include the tumour suppressor p110 retinoblastoma protein (pRB), and the cdc25 phosphatase. Evidence indicates that PP-1 dephosphorylates and inactivates cdc25 PTPase [72]. Therefore, blocking PP-1 activity would cause hyperphosphorylation and activation of cdc25, leading to the dephosphorylation and activation of cdc2/cyclin B kinase required for progression into M phase. This effect has been demonstrated in *Xenopus* extracts where inhibiting PP-1 activity causes activation of cdc2 kinase and premature entry into M phase [70]. Similarly, inhibition of PP-1 activity in synchronized hamster fibroblasts also activates cdc2 kinase and results in premature mitosis [73]. Conversely, addition of purified PP-1 to *Xenopus* extracts delays entry into mitosis.

The retinoblastoma protein (pRB) is a negative growth regulatory protein which most likely exerts its effects by controlling aspects of the cell cycle [74,75]. In this regard, it has been observed that pRB is hypophosphorylated in G_1 on serine and threonine residues and is hyperphosphorylated from S to M phase [76]. Injection of hypophosphorylated pRB into cells inhibits progression into S phase [77]. This underphosphorylated form of pRB binds and inhibits E2F transcription factors [78]. It is possible that pRB suppresses cell growth by blocking E2F-mediated transcription of genes necessary for S phase progression. Thus, phosphorylation of pRB would release E2F from pRB and permit transcription of needed cell cycle progression genes.

PP-1 may play a role in regulating the dephosphorylation of pRB that occurs during M phase such that the hyperphosphorylated form of pRB observed at the onset of M phase becomes hypophosphorylated in preparation for the following G_1 phase [79].

The dephosphorylation of pRB has been shown to commence during anaphase and continue until complete dephosphorylation in G_1 [80]. In this regard, it is interesting to note that the PP-1 mutants found in *A. nidulans* and *S. pombe* exhibit a mitotic block at anaphase. In addition, specific inhibitors of PP-1 inhibit the dephosphorylation of pRB during M phase and PP-1 dephosphorylates pRB *in vitro* [80,81]. It has also been demonstrated that PP-1 binds to pRB employing a modified yeast two-hybrid system [82] and *in vitro* utilizing purified proteins [81]. The relationship between PP-1 activity and the cell cycle shall be returned to later when we discuss the regulation of PP-1 by direct phosphorylation of the catalytic subunit.

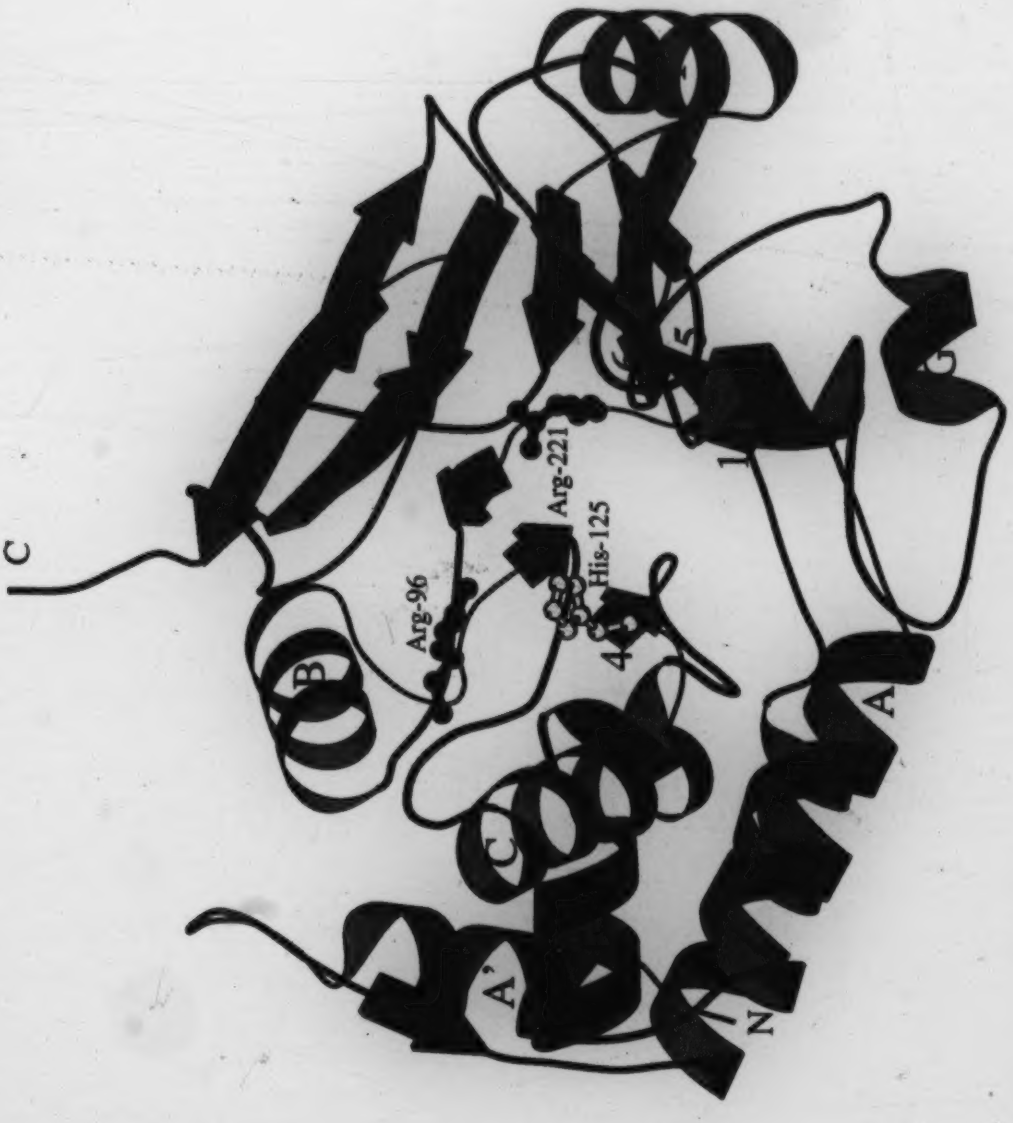
Catalytic Mechanism of PPP Phosphatases

While the work described in this thesis was being performed, crystal structures of two of the PPP phosphatases were determined [83-86]. The prediction that highly conserved catalytic core primary sequences translate into similar protein folding and catalytic mechanisms was borne out by comparing the crystal structures of PP-1 and PP-2B.

Both structures exhibit strikingly similar catalytic domain architecture consisting of a distorted β-sandwich of 11 β-strands surrounded on one side by 7 α-helices and on the other by 3 α-helices and a 3-stranded β-sheet (Fig. 1.10). The major difference between the two structures is the presence of the extended carboxy-terminal α-helix of PP-2B, completely lacking in PP-1, which contains the B subunit and calmodulin-binding domains in addition to the autoinhibitory domain of PP-2B (see Fig. 1.4).

Unlike protein serine/threonine kinases which possess an obvious catalytic cleft, the PPP family of protein phosphatases possess a shallow cleft on the surface of the molecule, enabling the relatively short pSer and pThr residues to reach the phosphate binding site, which includes two phosphate-binding arginine residues (Arg-96 and 221 in PP-1, Arg-122 and 254 in PP-2B). The primary structural feature of these phosphatases is a βαβαβ scaffold containing the amino acids of the phosphoesterase signature motif ($DXHX_nGGXXDX_nGNHD/E$), reviewed in [31]. All of the conserved amino acids of this sequence, except the Gly residues and final acidic residue, are directly involved in binding two metal atoms in the binuclear metal catalytic center (Fig. 1.11). The phosphoesterase signature motif is also found in other enzymes including kidney bean purple acid phosphatase (KBPAP), and *E. coli* exonucleases, sphingomyelin phosphodiesterase, and diadenosine tetraphosphatase. Significantly, the crystal structure of KBPAP has been determined and exhibits a binuclear metal catalytic core strikingly similar in architecture to those of the PPP protein phosphatase family [87].

Fig. 1.10 The crystal structure of rat protein phosphatase-1α [83]. The general conserved architecture consists of a central distorted β-sandwich, with β strands 2, 3, 4, 13 and 14 composing one sheet, offset by β strands 1, 5, 6, 10, 11 and 12 in the opposite sheet. This central feature is surrounded by 6 α helices (A to F) on one side and 3 α helices (G to I) and 3 β strands (7, 8 and 9) on the other. β strand 1' and α helix A' possess primary sequences uniqueto PP-1c and are therefore set apart from the designations. The remaining structures are conserved among PPP family phosphatases. The two phosphate-binding arginine residues (96 and 221) are indicated in dark blue while the catalytic His-125 is in yellow. The βαβαβ scaffold that contains the amino acids of the phosphodiesterase motif is comprised of β2, αB, β3, αC and β4.


C
Arg-96
B
Arg-221
His-125
44
1
5
G
A
C
A'
N

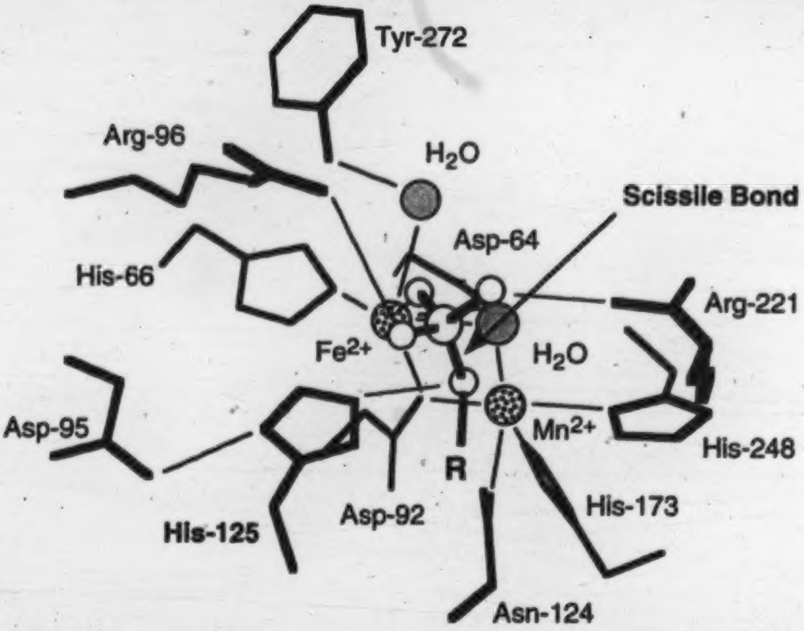


Fig. 1.11 The binuclear catalytic center of PP-1. Two metal ions are coordinated in the catalytic center of PP-1 by a host of residues and two water molecules, indicated by the black lines. The oxygen atoms of the target phosphate group are ligated by Arg-96, Arg-221, the Fe^{2+} of the recombinant enzyme and the catalytic His-125, indicated by the grey lines. The water molecule that bridges the two metal atoms is in-line with the phosphorus-oxygen scissile bond and acts as a nucleophile to attack the phosphorus atom. The catalytic His-125 donates a proton to the oxygen of the leaving group.

PP-2B binds one zinc and one iron atom in its catalytic center while the identities of the metals in native PP-1 are not fully known. In the recombinant PP-1 employed for crystal structure determinations, manganese and iron are present in the catalytic center. However, it is hypothesized that zinc is the second metal in native PP-1 [31].

It is believed that, unlike phosphatases of the protein tyrosine, dual-specificity, acid and alkaline classes which possess a two-step catalytic mechanism involving a phosphoryl-enzyme intermediate, the PPP family of phosphatases employs a mechanism of catalysis involving the direct transfer of phosphate to water, reviewed in [88,89]. Two molecules of water are bound in the catalytic center, one of which acts as a bridge between the two metal atoms. This water molecule is directly in-line with the scissile phosphorus-oxygen bond when substrate is bound and is believed to act as a nucleophile to attack the phosphorus atom of the phosphate group in an S_N2 mechanism. A histidine residue (125 in PP-1, 151 in PP-2B) opposite the attacking water is then in position to act as a general acid, donating a proton to the oxygen atom of the leaving serine or threonine residue. According to this catalytic mechanism, the metal atoms of the binuclear metal catalytic center contribute to catalysis by: 1. acting as Lewis acids to enhance the nucleophilicity of the attacking water molecule, 2. assisting in binding and orienting the substrate phosphate group and 3. stabilizing the transition state. This molecular mechanism is supported by biochemical evidence including severely compromised catalytic activity of mutants of the catalytic histidine and metal-binding residues. In addition, catalysis by the virtually identical KBPAP results in inversion of configuration of the product.

1.4 ■ REGULATION OF PROTEIN PHOSPHATASE-1 BY ASSOCIATION WITH OTHER PROTEINS

Clearly, PP-1 plays important roles in a broad diversity of critical physiological processes. The fact that PP-1 shares overlapping substrate specificity with PP-2A *in vitro* and that both of these phosphatases account for the majority of protein serine/threonine phosphatase activity in eukaryotic cells has prompted the fundamental question: How do only two members of the PPP phosphatase family specifically dephosphorylate the vast majority of substrates phosphorylated by protein serine/threonine kinases at the correct time and place without inappropriately disrupting protein phosphorylation homeostasis? As hinted at earlier, part of the answer lies in the finding that protein phosphatase catalytic subunits are associated with other proteins which change their catalytic efficiency or location.

PP-1 activity is regulated by interaction of the catalytic subunit, referred to hereafter as PP-1c, with endogenous regulatory subunit and inhibitor proteins (Fig. 1.12). Three acid and heat-stable inhibitors, inhibitor-1, DARPP-32, and inhibitor-2, have been identified. PP-1 activity is also targeted to glycogen particles by the G_M subunit in skeletal muscle and the G_L subunit in the liver. Similarly, PP-1c binds to the M subunit in muscle which targets the activity to myosin [90,91].

Inhibitor Proteins

As described in our discussion of the role of PP-1 in glycogen metabolism, inhibitor-1 (I-1) is phosphorylated by PKA to become an effective inhibitor of PP-1c [92,93]. However, I-1 is not efficiently dephosphorylated by PP-1, but is a good substrate for both PP-2A and PP-2B [94,95], suggesting the possibility of crosstalk between the PPP family of phosphatases through dephosphorylation of I-1. In addition, dephosphorylation of I-1

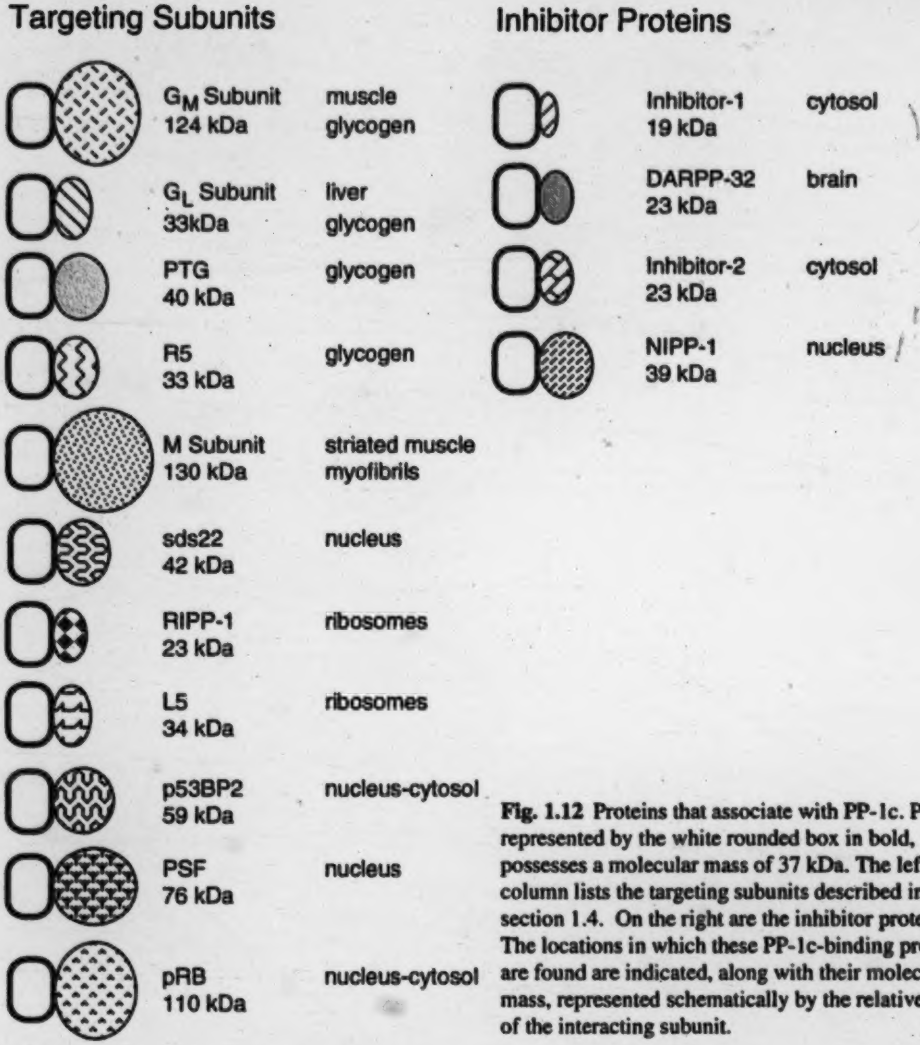

Targeting Subunits
G_M Subunit
124 kDa
muscle glycogen
G_L Subunit
33kDa
liver glycogen
PTG
40 kDa
glycogen
R5
33 kDa
glycogen
M Subunit
130 kDa
striated muscle myofibrils
sds22
42 kDa
nucleus
RIPP-1
23 kDa
ribosomes
L5
34 kDa
ribosomes
p53BP2
59 kDa
nucleus-cytosol
PSF
76 kDa
nucleus
pRB
110 kDa
nucleus-cytosol
Inhibitor Proteins
Inhibitor-1
19 kDa
cytosol
DARPP-32
23 kDa
brain
Inhibitor-2
23 kDa
cytosol
NIPP-1
39 kDa
nucleus

Fig. 1.12 Proteins that associate with PP-1c. PP-1c, represented by the white rounded box in bold, possesses a molecular mass of 37 kDa. The left column lists the targeting subunits described in section 1.4. On the right are the inhibitor proteins. The locations in which these PP-1c-binding proteins are found are indicated, along with their molecular mass, represented schematically by the relative size of the interacting subunit.

by PP-2B may provide a mechanism for the antagonism of cAMP signaling by increases in intracellular Ca^{2+} concentrations. The widespread tissue distribution of I-1 implies a role for this inhibitor in numerous physiological processes [96].

DARPP-32 is related to I-1 both structurally and functionally [97,98]. This endogenous inhibitor is found in the brain and, like I-1, is activated after phosphorylation by PKA. DARPP-32 is specifically dephosphorylated by PP-2B [97,99,100], which is localized in the same regions of the brain.

Inhibitor-2 (I-2), also known as the modulator protein, is the third endogenous heat and acid-stable inhibitor of PP-1c. I-2 shares no sequence similarity to I-1 and

DARPP-32 [101] and, unlike its counterpart inhibitor proteins, does not require prior phosphorylation to be active. I-2 causes the rapid inhibition of PP-1c and a time-dependent conformational change of PP-1c that leads to inactivation of the enzyme [56]. However, when the inactive PP-1c-I-2 complex (known as PP-1I) is phosphorylated on I-2 by glycogen synthase kinase-3 (GSK-3), reactivation of the PP-1 activity has been observed. However, it is not believed that this event occurs *in vivo*, since mass spectrometric analysis of I-2 purified in the presence of phosphatase inhibitors indicated that the site targeted by GSK-3 is not phosphorylated [102]. The intracellular level of I-2 in rat fibroblasts oscillates during the cell cycle, peaking during S and M phases, suggesting a role for I-2 inhibition of PP-1 activity during the cell cycle [103].

Recently, another class of endogenous inhibitor proteins has been isolated from bovine thymus cells, where high levels of PP-1 are associated in the nuclear fraction in an inactive form that is tightly bound to chromatin and the nuclear matrix [104]. The inhibition of this nuclear form of PP-1 is due to the association of one of two small nuclear proteins, referred to as NIPP-1 or 2 [105,106]. These are very potent and specific inhibitors of PP-1c which, in contrast to I-1 and DARPP-32, are inactivated as inhibitors of PP-1 after phosphorylation by PKA [107]. Most recently, NIPP-1 has been shown to bind specifically to uracil-rich sequences of RNA, suggesting that NIPP-1 also serves to target PP-1 to RNA [108].

Targeting and Regulatory Subunits

The paradigm for regulation of PP-1 by targeting or regulatory subunits is the interaction of PP-1c with the glycogen-binding G_M subunit [91]. This example illustrates how restricting the location of the catalytic subunit of PP-1 profoundly influences its activity toward different subsets of substrates.

Originally isolated from skeletal muscle, the glycogen-associated form of PP-1, referred to as PP-1G_M, consists of PP-1c and the 124 kDa G_M subunit [109,110]. This form of PP-1 and the substrates of PP-1 that influence glycogen metabolism (glycogen synthase, phosphorylase kinase, and phosphorylase, discussed previously in section 1.3) bind glycogen with high affinity. The glycogen-bound form of PP-1 is 5 to 8 times more active towards phosphorylase than free PP-1c [111]. This is explained by lower K_M resulting from the co-localization of the enzyme and substrate to the surface of the glycogen molecule, where interaction of the molecules is restricted from three-dimensional space to a two-dimensional surface (Fig. 1.13).

Dissociation of PP-1c from the G_M subunit results in concomitant reduction in phosphatase activity toward PP-1 substrates bound to glycogen that are involved in

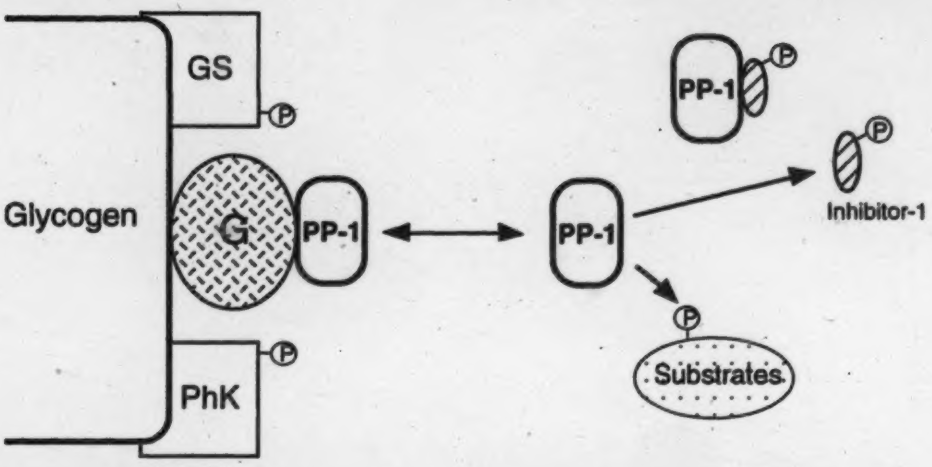


Fig. 1.13 The effect of co-localization on PP-1 activity. Targeting of PP-1 to glycogen particles results in increased activity toward glycogen-bound substrates (GS: glycogen synthase, PhK: phosphorylase kinase) by restricting PP-1 to the same location. PP-1 free in the cytosol has reduced interactions with glycogen-bound substrates and increased interactions with cytosolic substrates and inhibitor proteins.

glycogen metabolism [59]. This is part of the mechanism whereby PKA reduces the dephosphorylation of glycogen synthase and phosphorylase kinase by PP-1 in response to adrenalin.

Thus, targeting subunits localize PP-1c and thereby restrict the substrates with which it can effectively interact. While in the PP-1G_M form, PP-1 possesses increased activity toward substrates within its vicinity on the glycogen particle and reduced activity toward other substrates found in other subcellular locations. Likewise, sequestering PP-1c to glycogen particles reduces its sensitivity to cytosolic endogenous inhibitors by restricting its interaction with these proteins [109].

In addition, PP-1G_M also associates with the sarcoplasmic reticulum through a different region of the G_M subunit [112,113]. Thus, a single targeting subunit may be responsible for multiple compartmentalization of a single enzymatic activity. In this case, the likely target of PP-1G_M is phospholamban [114]. In response to adrenalin, PP-1 activity is reduced through the action of PKA as discussed previously, resulting in increased phosphorylation of phospholamban. This in turn results in increased uptake of Ca^{2+} into the sarcoplasmic reticulum and relaxation of muscle.

A form of glycogen-associated PP-1 has also been isolated from liver, where PP-1c is localized to glycogen particles through the G_L subunit, a 33 kDa protein that is distinct from the G_M subunit [115,116]. This form of PP-1, known as PP-1G_L, exhibits greater activity toward its glycogen-bound substrates and is regulated allosterically by a mechanism that does not involve PKA [117]. In the case of PP-1G_L, activity is controlled by low concentrations of phosphorylase in the liver.

Recently, two related PP-1 binding proteins that associate with glycogen, termed PTG and R5 have also been identified. PTG (protein targeting to glycogen) is expressed mainly in insulin-sensitive tissues and binds glycogen synthase, phosphorylase kinase, and phosphorylase, in addition to binding PP-1c [118]. As such PTG is thought to serve as a molecular scaffold on which protein interactions involving PP-1 and glycogen metabolism may be facilitated. R5 is a 36 kDa protein that, like PTG, is found in a wide variety of tissues and is regulated by a mechanism that is distinct from both PP-1G_M and PP-1G_L [119]. Like PP-1 bound to the G_L subunit, R5 alters the affinity of PP-1 for its glycogen-bound substrates.

Another type of interacting subunit targets PP-1c to the myofibrils of striated and smooth muscle. This form of PP-1, referred to as PP-1M, dephosphorylates the phosphorylated light chain of myosin and consists of a heterotrimer containing PP-1c, a 130 kDa PP-1-binding M subunit, and a 20 kDa protein [120-124]. When bound to myofibrils, PP-1M increases the rate of myosin dephosphorylation leading to muscle relaxation, while diminishing the activity of PP-1c toward glycogen-bound substrates by restricting its location. In permeabilized smooth muscle, arachidonic acid releases PP-1c from the M subunit resulting in reduced myosin dephosphorylation by abolishing the compartmentalization effect [125].

PP-1 is also enriched in the nucleus of cells where it co-localizes with chromosomes at mitosis, implying the existence of nuclear localization targeting subunits of PP-1c. sds22 in *S. pombe* was identified as a 30 kDa nuclear protein that interacts with PP-1 and was shown to be essential for the metaphase-anaphase progression during mitosis [126,127]. It is believed that sds22 represents a nuclear targeting subunit of PP-1. Recently, the rat homolog of sds22 (41.6 kDa) was isolated in a 260 kDa complex of PP-1 from rat hepatocytes, termed PP-1N_{sds22}, and was shown to inhibit PP-1 activity [128]. This is similar to the situation with the NIPP inhibitor proteins which are also present as nuclear inhibitors of PP-1c and may represent another class of nuclear PP-1c targeting subunits.

Yet another class of interacting proteins has been recently isolated from ribosomes. The ribosome-associated PP-1, termed PP-1R, was inhibited by a 23 kDa basic protein called RIPP-1 [129]. Like I-2, RIPP-1 causes a time-dependent conformational change of PP-1c into a less active form. Recently, the ribosomal protein L5 [130] was shown to bind PP-1c directly and is believed to represent a ribosomal targeting subunit [131]. It is already known that PP-1 dephosphorylates the ribosomal S6 protein *in vivo* [132], and hence it is logical that a ribosomal targeting subunit, such as L5, exists.

Structural Basis for PP-1c Binding of Associated Proteins

Each of the heterologous forms of PP-1 characterized thus far contain only one PP-1c-binding subunit, suggesting that different interacting subunits utilize identical or overlapping binding sites on PP-1c and possess a common PP-1c-binding motif. In support of this hypothesis, it has been observed that different targeting subunits compete for PP-1 binding, the competition being measured by changes in PP-1 activity toward substrates from different compartments depending on the targeting subunit being analyzed.

A sequence alignment of the different G subunits revealed a short, highly conserved region which, when synthesized as a peptide fragment, was shown to bind to PP-1c [133]. The recently described crystal structure of a peptide fragment of the G_M subunit bound to PP-1c has shed considerable light on the PP-1c-binding motif of several proteins and how they interact with PP-1c [134]. The G_M peptide binds PP-1c in a region that is distal from the catalytic site, thereby permitting catalysis to continue when targeting subunits are bound and explaining the observation that affinity chromatography which anchors PP-1c in its catalytic site can be used to isolate PP-1c-binding proteins [135,136]. The amino acids that constitute the area of PP-1c that binds the G_M peptide are highly conserved throughout all isoforms and homologs of PP-1c, but are not found in PP-2A or PP-2B.

The region of PP-1c that binds the G_M peptide consists of a hydrophobic groove anchored by an acidic pocket. The PP-1c-binding motif of the G_M peptide is a short stretch consisting of Arg-Val-Ser-Phe. The Phe and Val residues contribute the majority of binding energy by binding in the hydrophobic groove in an extended peptide conformation, while the arginine residue forms ionic interactions within the acidic pocket. Comparison of several PP-1c-binding proteins revealed the presence of a consensus binding motif consisting of Arg/Lys-Val/Ile-Xaa-Phe. An alignment of all known PP-1c-binding proteins is shown in Table 1.4. Most of the proteins described in this section possess a variation of this binding motif. Mutations in the PP-1c- binding motif of many of these proteins results in marked reduction in their ability to affect PP-1 activity, most likely through loss of binding potential. Two of the proteins listed, PSF [137] and p53BP2 [138], have been recently identified and the outcomes of these interactions are not fully understood. PSF is a splicing factor which binds polypyrimidine regions of mammalian introns and plays a role in the formation of

Table 1.4 Alignment of the PP-1c binding motifs of proteins that associate with PP-1c. Indicated at the right is the range of amino acids listed for each protein. The Arg/Lys-Val/Ile-Xaa-Phe motif is found in all the proteins listed except the final three. An alanine residue in the second amino acid position of the motif found in the L5 protein is sterically permitted, while the leucine residue of pRB and arginine residue of I-2 are too large to fit into the hydrophobic pocket of PP-1c that is part of the binding domain.

Protein	Binding Domain	
G_M subunit	VHTASSGGRRVSFADNFGFL	56-75
G_L subunit	TVQEKKVKKRVSFADNQGLA	52-71
PTG	KSPHNQAKKRVVFADSKGLS	52-71
R5	KCSHNQAKKRVVFADSKGLS	75-94
PSF	DTPMRGRQLRVRFATHAAAL	7-26
NIPP-1	RPKRKRKNSRVTFSEDDEII	191-210
p53BP2	GSERIAHGMRVKFNPLALLL	313-322
M subunit	PPVVKRQKTKVKFDDGAVFL	26-45
I-1	MEQDNSPRKIQFTVPLLEP	1-19
DARPP-32	MDPKDRKKIQFSVPAPPS	1-18
L5	MGFVKVVKNKAYFKRYQVKF	1-20
pRB	GSNPPKPLKKLRFDIEGSDE	865-884
I-2	DLSPEEREKKRQFEMKRKLH	137-146

spliceosomes [139] while p53BP2 binds the tumour suppressor protein p53, suggesting further roles for PP-1 associated with these proteins.

The role of reversible protein phosphorylation as a mechanism of PP-1 regulation will be discussed in detail in section 1.5. However, the structural aspects of the regulation of PP-1 through phosphorylation of targeting subunits will be introduced here. As illustrated in Table 1.4, the variable amino acid in the PP-1c-binding motif of the G_M subunit is a serine that is part of a PKA consensus sequence (see Table 1.1) that overlaps the binding motif. It is the phosphorylation of this site by PKA in response to adrenalin that causes the dissociation of PP-1c from the G_M subunit, thereby reducing the activity of PP-1 towards its glycogen-bound substrates, as described previously. The formation of a pSer at this site causes steric hindrance and introduces negative charge in the vicinity of the acidic pocket, thereby abolishing binding of the phosphorylated G_M subunit.

The NIPP inhibitor proteins are phosphorylated by PKA and casein kinase-II (CK-II) resulting in dramatic reduction in their inhibitory potency [107,140]. Inspection of the primary sequence surrounding the PP-1-binding motif of NIPP-1 reveals the presence of PKA and CK-II consensus sequences (see Table 1.1) flanking the binding motif. Phosphorylation of either of these sites would result in reduction in binding potential of the phosphorylated NIPP-1. Therefore, the inhibition of NIPP-1 as a PP-1 inhibitor by the actions of PKA or CK-II is explained by phosphorylation near the PP-1 binding motif. This is in contrast to the situation with I-1 and DARPP-32 which are activated upon phosphorylation by PKA. In this case, the phosphorylation site is distal to the PP-1 binding motif and is hypothesized to bind at the catalytic site in an autoinhibitory fashion.

Two of the proteins that bind to PP-1c listed in Table 1.4, pRB [141] and I-2 [101], contain sequences that do not strictly conform to the PP-1c- binding motif. In both cases, the amino acid that substitutes in the motif Val/Ile position is larger than the permitted residues, which can not be accommodated by the binding groove on PP-1c according to the crystallographic data. It is likely that pRB and I-2 derive some weak binding energy through partial interaction between their imperfect PP-1 binding motifs. However, for both pRB and I-2 to productively interact with PP-1c, they must employ an additional mode of interaction with PP-1c, suggesting that other regions of PP-1c may recognize and bind proteins. This hypothesis is supported by the observation that the only proteolytic fragment of I-2 to retain any inhibitory activity consists of amino acids 25-114 [101], which does not contain the partial PP-1c-binding motif.

With the recent development of techniques designed to identify protein-protein interactions, such as the two-hybrid system [82] and PP-1 affinity chromatography [135,136], the number of proteins found to interact with PP-1c has been growing rapidly during the time of the work described in this thesis. It is becoming clear that the ability to restrict the subcellular location of PP-1 is a significant strategy to regulate its activity toward different subsets of substrates. Considering that PP-1 is involved in a wide variety of physiological roles, it is likely that there are several uncharacterized subunits that regulate PP-1 activity under specific circumstances. Research in this area is very intense and in the future will yield considerable appreciation of the intricacies of PP-1 regulation in numerous biochemical processes in addition to uncovering a number of novel physiological substrates of PP-1.

Although targeting PP-1 to discrete subcellular locations is emerging as a major means of regulating PP-1, evidence suggests that PP-1 is also regulated by other mechanisms. It is becoming clear that PP-1 is regulated by reversible protein phosphorylation and a growing number of naturally occurring toxins. Understanding the molecular mechanisms underlying these two modes of PP-1 regulation is the objective of the work outlined in this thesis.

1.5 ■ REGULATION OF PROTEIN PHOSPHATASE-1 BY REVERSIBLE PROTEIN PHOSPHORYLATION

The activity of PP-1 can be regulated through the effects of reversible protein phosphorylation. However, two modes of regulation have emerged. First, the most widely studied mechanism involves the phosphorylation of targeting or regulatory subunits and proceeds through a protein intermediate. The second mode is via direct phosphorylation of the catalytic subunit itself.

Indirect Phosphorylation

The best example of this is the regulation of the interaction of the G_M targeting subunit with PP-1c by phosphorylation. The G_M subunit can be phosphorylated on two sites, classically referred to as Site 1 and Site 2, each manifesting opposite effects on the catalytic efficiency of PP-1 on glycogen-bound substrates [30,91]. The phosphorylation of Site 1 by ISPK (discussed in section 1.3) results in an increase in the activity of PP-1 toward its glycogen-bound substrates and a subsequent increase in glycogenesis in response to insulin [59]. Conversely, as discussed above, phosphorylation of Site 2 by PKA in response to adrenalin causes dissociation of PP-1c from the G_M subunit, resulting in reduced activity towards its glycogen-bound subunits.

Other examples of regulation of PP-1 through indirect phosphorylation have already been discussed. These include the phosphorylation and activation of I-1/DARPP-32 by PKA, and the phosphorylation and inactivation of NIPP inhibitor proteins by PKA and CK-II.

The potential for crosstalk between the phosphatases exists when other PPP family phosphatases dephosphorylate regulatory sites on targeting or regulatory subunits. For example, PP-2B activity commonly antagonizes the activity of PKA and does so specifically by dephosphorylating PKA phosphorylation sites on the G_M targeting subunit and I-1/DARPP-32. In this manner, Ca^{2+} signaling can oppose cAMP-induced signaling.

Direct Phosphorylation by Protein Tyrosine Kinases

To this point, we have discussed regulation of PP-1 by interactions with other proteins and the phosphorylation of those interacting proteins. The concept that PP-1c itself might be regulated directly by phosphorylation has been slow to gain favour. First, biochemists had to overcome the counterintuitive idea that if phosphorylation resulted in inactivation of phosphatases, then loss of phosphatase activity would occur exponentially, resulting in the stable phosphorylation of many proteins. Alternatively, if phosphorylation resulted in activation of phosphatases, then phosphatases would remove phosphates as quickly as they were bound and this was considered counterproductive. Second, the ability to isolate phosphorylated phosphatases requires potent and rapid inhibitors of phosphatase activity, such as okadaic acid (discussed in section 1.6), something that was not widely available until the last decade. In addition, because of the use of these inhibitors, activity and phosphorylation of phosphatases can not be analyzed in the same experiment. Thus, significant theoretical and practical obstacles existed to hinder the exploration of the hypothesis that PP-1c might be regulated by direct phosphorylation [142]. However, recent advances have demonstrated that PP-1c is indeed phosphorylated and that this event has regulatory effects on PP-1c activity.

The first report of phosphorylation of PP-1c occurred in 1986 when it was shown that PP-1c could be phosphorylated by the v-src protein tyrosine kinase to a maximal stoichiometry of approximately 0.3 mole phosphate per mole PP-1c, resulting in about 30% reduction in activity [143]. Proteolysis of a C-terminal fragment of PP-1c by trypsin removed the site of phosphorylation, but the precise location of the covalent modification was not determined [144]. Later experiments demonstrated that overexpression of v-src in cells results in a three-fold reduction of PP-1 activity toward the ribosomal S6 protein [145]. However, it was discovered that phosphorylation of interacting proteins is responsible for regulating the loss of phosphatase activity rather than phosphorylation of PP-1c itself. Therefore, there is no evidence to suggest that regulation of PP-1c by direct phosphorylation by v-src occurs *in vivo*.

Recently, PP-1c has also been shown to be phosphorylated on the C-terminal by the v-abl protein tyrosine kinase. This reaction proceeded to a stoichiometry of 0.7 mol/mol for cytosolic forms of PP-1 and 0.07 mol/mol for PP-1G [146]. In contrast to phosphorylation by v-src, phosphorylation by v-abl had no effect on the activity of PP-1 toward phosphorylase. However, the ability of I-2 to bind PP-1c was abolished upon v-abl phosphorylation and v-abl was unable to phosphorylate PP-1I (PP-1c bound to I-2), suggesting that the phosphorylation site is part of or near the site bound by I-2.

It has been suggested that the site of v-abl and v-src phosphorylation is Tyr-306; however, precise verification of this hypothesis has not been carried out. With recent advances in recombinant expression of the various isoforms of PP-1c, this question could be addressed due to the variability in the C-terminal primary sequence of the various isoforms. Both the α and β isoforms of PP-1c contain a tyrosine at position 306 with the β isoform possessing an additional tyrosine residue at position 308 (see Table 1.3). Conversely, the γ isoform has no tyrosine residues in its C-terminal. Parallel phosphorylation reactions containing purified isoforms of PP-1c and v-src or v-abl could be analyzed by autoradiography or anti-pTyr antibodies for the presence of pTyr on the various isoforms. Then, by process of elimination, the precise site of PTK phosphorylation could be determined. Phosphorylation on only the β isoform implicates Tyr-308 as the PTK site, whereas phosphorylation of both α and β isoforms suggests that Tyr-306 is indeed the target of PTK action. Phosphorylation of all three isoforms by v-abl or v-src would suggest a phosphorylation site farther from the C-terminal which is lost upon trypsin digestion. It is tempting to imagine crosstalk between pTyr signaling and pSer signaling given the phosphorylation of PP-1 by v-src or v-abl, but unfortunately, there is no strong evidence to suggest that this phenomenon occurs *in vivo*.

Direct Phosphorylation by Protein Serine/Threonine Kinases

The threonine residue at position 320 of the C-terminal tail present in all three isoforms of PP-1c has most recently been identified as another site of phosphorylation of the catalytic subunit. Active cdc2 kinase bound to cyclin A or B phosphorylated this residue within the consensus sequence Thr-Pro-Pro-Arg in the C-terminal tail of mammalian PP-1α and PP-1γ (see Table 1.3) to a stoichiometry of 0.5 mol/mol, resulting in a 50% loss of activity [147]. In the presence of potent phosphatase inhibitors, a 1.5-fold increase in the level of phosphorylation at this site was observed, suggesting that auto-dephosphorylation of this site occurs. Mutation of this site to alanine in PP-1α resulted in the loss of PP-1c phosphorylation by cdc2 kinase.

A peptide fragment of PP-1α containing the cdc2 kinase phosphorylation site was an excellent *in vitro* substrate for cdc2 kinase, as well as the p42 and p44 MAP kinases. However, this peptide was not phosphorylated by PKA, CK-II, PKC, or phosphorylase kinase. It was subsequently shown that MAP kinases phosphorylate PP-1α to low stoichiometry *in vitro* with no change in phosphatase activity, suggesting that MAP kinases may not phosphorylate PP-1 *in vivo* [142].

Work describing the cdc2 kinase-mediated phosphorylation of an isoform of PP-1 from the fission yeast *S. pombe*, referred to as dis2, was published at the same time as the work described above outlining the phosphorylation of mammalian forms of PP-1 [148]. Two genes encoding different isoforms of PP-1 exist in *S. pombe*: *dis2*$^+$ contains the cdc2 kinase consensus sequence, while *sds21*$^+$ does not. In the work done with *S. pombe* the phosphorylation of dis2 was observed both *in vitro* and *in vivo*. The *in vitro* reaction proceeded to a stoichiometry of about 0.7 mol/mol and resulted in a 70% loss of activity.

Since the cdc2 kinase is a key cell cycle regulatory element and accumulating evidence suggests that PP-1 also plays important roles in this vital process (discussed in section 1.3), it was of interest to examine the interaction between these two enzymes within the context of the cell cycle of *S. pombe*. When a threonine to alanine substitution mutant of dis2, not phosphorylated by cdc2 kinase *in vitro*, was expressed in *S. pombe* cells that lack the wild type *dis2*$^+$ gene, inhibition of colony formation occurred. It is believed that the inhibition of dis2 by cdc2 kinase at the onset of mitosis, when cdc2 kinase activity is high, assists in the progression into M phase. As mitosis progresses, cdc2 kinase activity is reduced at the metaphase-anaphase transition. At this point, auto-dephosphorylation of dis2 may occur such that dis2 activity is restored, directing progression into anaphase. In addition, activated dis2 may also dephosphorylate and inactivate the cdc25 PTPase (discussed in section 1.3, see Fig. 1.9), thereby facilitating hyperphosphorylation and inactivation of cdc2 kinase until conditions are appropriate for mitosis to occur again.

In support of this hypothesis, the phosphorylation of PP-1 in rat fibroblasts is maximal during early to mid-mitosis [149]. The level of phosphorylation of PP-1 decreases with the addition of the cdc2 kinase inhibitor, olomoucine [142], and increases with the addition of the potent PP-1 inhibitor okadaic acid, supporting the evidence that PP-1 is phosphorylated by cdc2 kinase and auto-dephosphorylates. Phosphorylated PP-1 is also found almost exclusively in non-chromosomal regions, based on immunofluorescence microscopy of intact cells.

Given the evidence outlined above, it is clear that the catalytic subunit of PP-1 itself is phosphorylated and regulated directly by the action of protein kinases and that at least one of these phosphorylation events occurs *in vivo*. Thus, the initial bias against the concept of direct phosphorylation of PP-1c was unfounded and a new area of research aimed at understanding the regulation of PP-1c by direct phosphorylation has emerged. Preliminary data from *S. pombe* suggests the presence of pSer in PP-1 that does not possess the cdc2 kinase phosphorylation site isolated from intact cells [148]. Therefore, there must exist another site(s) of phosphorylation on PP-1 in *S. pombe*. Given the extreme degree of sequence conservation of PP-1 throughout evolution, it is likely that PP-1 from other organisms is also phosphorylated on sites other than the cdc2 kinase consensus sequence. Chapter 4 of this thesis outlines experiments designed to examine the phosphorylation by PKA of one such site on PP-1c from diverse lower eukaryotes, including *S. pombe*.

1.6 ■ REGULATION OF PROTEIN PHOSPHATASE-1 BY MARINE TOXINS

Given the diversity of indispensable physiological processes in which PP-1 plays a regulatory role, the inappropriate inhibition of PP-1c can have detrimental systemic effects. Within the last decade, it has become clear that a number of natural product toxins from marine sources exert their effects through direct and potent inhibition of PP-1c [150-152]. Among these are related cyclic peptide toxins from cyanobacteria known as microcystins and nodularins and a polyether fatty acid termed okadaic acid, first isolated from marine sponges.

Okadaic Acid

Twenty years ago, a form of gastroenteritis that results from eating mussels was first identified and designated as diarrhetic shellfish poisoning (DSP) [153]. Although the first scientific report of this condition has been relatively recent, it is believed that the condition now known as diarrhetic shellfish poisoning has existed for centuries in Japanese folklore where it is thought that mussels may be poisonous during the season of the paulownia flowers, which is between the months of June and July. Diarrhetic shellfish poisoning is a serious human health hazard and occurs worldwide, especially in countries where shellfish are a regular part of the diet, such as Japan. Symptoms of those suffering from diarrhetic shellfish poisoning are mainly gastrointestinal problems such as diarrhea, nausea, vomiting, and abdominal pain. These symptoms generally are manifested quickly, within 30 minutes of consuming intoxicated mussels. Bouts of diarrhea and vomiting generally occur four or five times over the course of the poisoning, which lasts three days on average. However, severe cases of diarrhetic shellfish poisoning have been documented as causing up to 20 bouts of vomiting and diarrhea a day!

The causative agent of diarrhetic shellfish poisoning was isolated and characterized soon after the first report of the condition [154]. The compound, termed dinophysistoxin-1, was shown to be the 35*S*-methyl variant [155] of a recently characterized cytotoxic 38-carbon polyether fatty acid isolated from the marine sponges *Halichondria okadaii* and *Halichondria melanodocia*, called okadaic acid after one of the sponges [156] (Fig. 1.14). It was observed that mussels became toxic upon feeding on the marine dinoflagellate *Dinophysis fortii*, from which the name dinophysistoxin was derived. Since other dinoflagellates, in particular the *Prorocentrum* species, were also

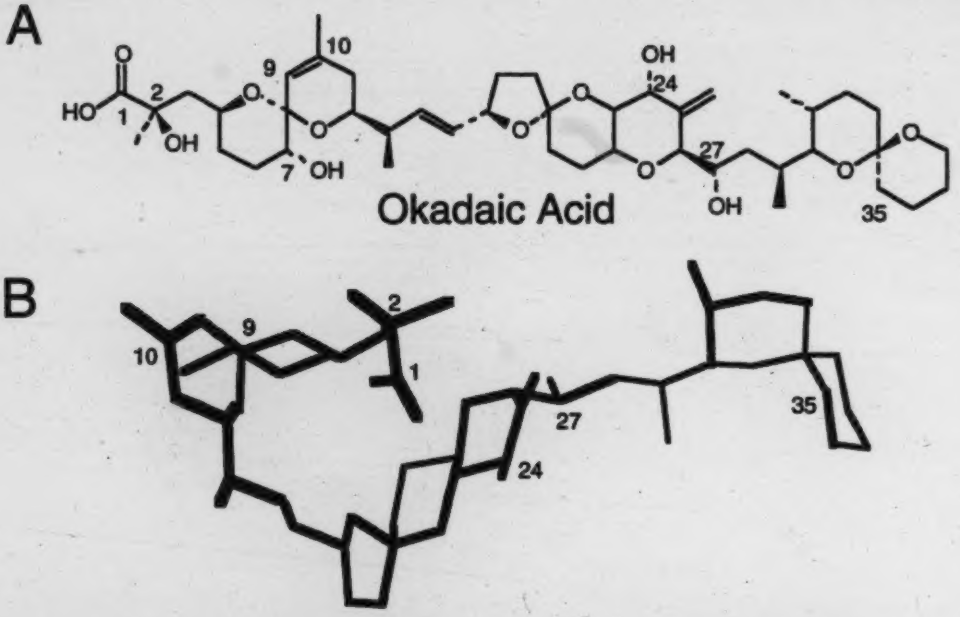


Fig. 1.14 Okadaic acid. **A**. Linear structure of okadaic acid. **B**. Crystal structure of okadaic acid. Selected carbon atoms are denoted by their linear structure location. Note that the carboxy group of okadaic acid bends back on itself to form a pseudo-ring structure with a hydrophobic tail reminiscent of the cyclic peptide marine toxins (see Figs. 1.15 and 1.16).

known to be toxic [157,158], the nature of that toxicity was investigated and it was soon discovered that *Prorocentrum lima* was a primary producer of okadaic acid and that this toxin also causes diarrhetic shellfish poisoning [159].

It wasn't until 1987 that it was realized that okadaic acid potently and specifically inhibits PP-1 and PP-2A [160], with the IC_{50} for the toxin being two orders of magnitude lower for PP-2A inhibition (IC_{50} of 0.2 nM) than PP-1 (IC_{50} of 20 nM) [161]. Despite sharing similar protein folding and catalytic mechanism, PP-2B is inhibited to a much lesser extent (IC_{50} of 5 μM). Subsequently, okadaic acid has also been shown to be a powerful tumour promoter [162,163]. Structural modification studies with okadaic acid have indicated the influence of several hydroxyl groups throughout the structure at C-2, C-7, C-24, and C-27 on the ability of okadaic acid to inhibit the phosphatases [164], suggesting that these structural elements play important roles in the interaction of the toxin with its target.

Being hydrophobic and cell permeable [165], the differential inhibition of PP-1 and PP-2A activity in intact cells by okadaic acid has made this marine toxin an indispensable research tool for examining phosphatase-mediated processes *in vivo* [166].

Presently, okadaic acid is a common component of protein kinase assays to inhibit contaminating phosphatase activity.

Cyclic Peptide Inhibitors

Blue-green algae, also known as cyanobacteria, are not true algae but rather photosynthetic bacteria that are believed to be the first form of life to exist on land. As such, the biochemistry of cyanobacteria is thought to be a reflection of their early evolutionary origin. Cyanobacteria in surface water have been associated with toxic effects on wildlife and domesticated animals [167]. The first scientific report of the toxic effects of cyanobacteria was in 1878 in Australia when a bloom of cyanobacteria in a freshwater lake was linked with terrible deaths of farm animals [168]. Since that time, reports of cyanobacterial intoxication of animals have been reported worldwide, with death generally being associated with severe liver damage, among other effects. Adverse effects upon human health upon exposure to water contaminated with cyanobacteria have also been reported worldwide [169]. In Australia, a sickness known as Barcoo fever is now believed to be caused by ingestion of cyanobacteria. Typical symptoms include diarrhea and vomiting, which are also common effects observed among farm animals exposed to cyanobacteria. Elevated liver enzyme levels in the blood have also been associated with cyanobacterial exposure, an indicator of liver damage [170]. In February of 1996, 50 patients who underwent kidney dialysis at the Kidney Disease Institute in Caruaru, Brazil, died as a result of toxic hepatitis caused by cyanobacterial toxins in the dialysis water [171]. Obviously, cyanobacterial toxins are a major health concern, especially in areas of the world where surface water exists under conditions that are optimal for cyanobacterial blooms.

In the early 1980s, the primary structure of one of the cyanobacterial toxins that cause such drastic hepatotoxic effects was determined [172]. This was a cyclic peptide containing seven amino acids, referred to as microcystin. Subsequently, other cyclic peptide toxins containing five amino acids were identified and termed nodularins. These compounds were named after the cyanobacteria from which they were isolated, being members of the *Microcystis* and *Nodularia* genera. The toxins are thought to enter the liver via bile salt transport where they are known to cause reorganization of hepatocyte cytoskeletal components [173,174], resulting in the formation of hepatic lesions. Blood seeps into the liver, enlarging it. Hepatocyte cell contents spill into the blood and, in extreme cases, hepatocytes become dislodged and freely flow in the bloodstream.

Both microcystins and nodularins have been characterized as powerful tumour promoters, much like okadaic acid [175-178]. Also like okadaic acid, microcystins and nodularins were discovered to be potent inhibitors of PP-1c and PP-2Ac [179-181], save that in this case both cyclic peptide inhibitors are equipotent against PP-1 and PP-2A, with IC_{50} values being 0.2 nM for microcystins and 0.2 nM for nodularins.

Microcystins and nodularins are chemically distinguished by the presence of a unique 20-carbon β-amino acid referred to as Adda ([2S, 3S, 8S, 9S]- 3 -amino - 9 -methoxy - 2,6,8, -trimethyl -10-phenyldeca-4,6,-dienoic acid) (Fig. 1.15) [182]. Microcystins differ in the nature of two variable L-amino acids indicated by suffix letters. For example, the most commonly isolated microcystin is microcystin-LR, which contains leucine and arginine in the variable amino acid positions. In addition, variations of microcystins occur as a result of the absence of methyl groups on the D-erythro-β-methyl aspartic (Masp) acid and/or the N-methyldehydroalanine (Mdha) residues. To date, over 50 distinct microcystins have been isolated and characterized [150,151,183].

Nodularins are cyclic pentapeptides that also contain the characteristic Adda amino acid (Fig. 1.16). In addition, nodularins also contain Masp and N-methyldehydrobutyrine (Mdhb). Nodularins contain one variable L-amino acid, indicated by a suffix letter. Only two nodularins are known, being nodularin-R, the first nodularin isolated containing arginine in the variable position, and nodularin-V, also known as motuporin, which was isolated from the marine sponge *Theonella swinhoie grey* and was named after the Papua New Guinea island of Motupore where the sponges were collected [184].

Although microcystins and nodularins have been extensively characterized in fresh and brackish water environments, they have recently been isolated in the marine environment [185], raising the likelihood that these cyclic peptide hepatotoxins are a contributor to marine-related illnesses. Microcystins were found to be the causative agent of netpen liver disease among Atlantic salmon farmed in the Pacific Northeast [186,187]. This disease has had devastating effects on farmed salmon and has caused the loss of millions of dollars in the fish farming industry of British Columbia and the state of Washington. Thus, microcystins and nodularins are also similar to okadaic acid in being present in the marine environments and together form the group of marine toxins examined in this thesis.

Significant biochemical differences exist between microcystins and nodularins. Although both rapidly bind and potently inhibit PP-1 and PP-2A, only microcystins form a covalent linkage with PP-1c or PP-2Ac after hours of incubation [188,189]. It has been demonstrated that microcystins form this covalent link between the Mdha residue of microcystins and Cys-273 of PP-1c, placing microcystins very close to the catalytic center of PP-1c (recall that Tyr-272 coordinates one of the catalytic center water

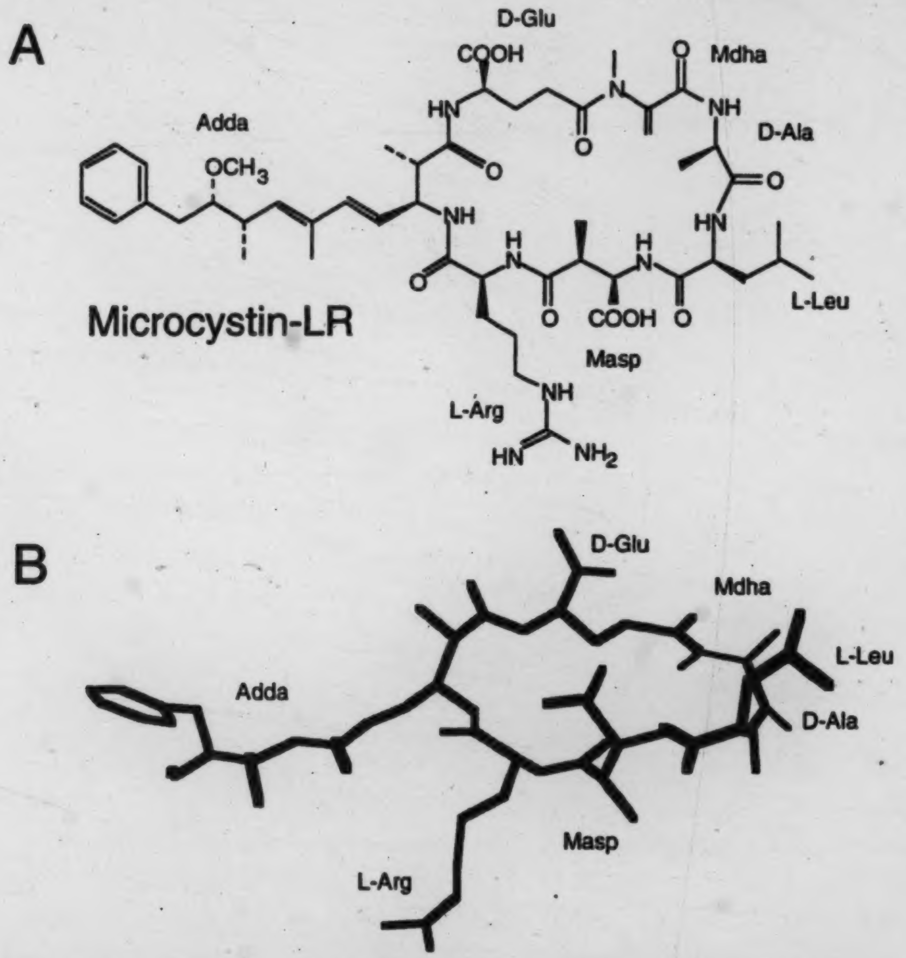

A
D-Glu
COOH
O
Mdha
Adda
HN
N
NH
D-Ala
OCH3
O
O
O
NH
HN
H
N
H
N
L-Leu
O
Microcystin-LR
O
COOH
O
Masp
NH
L-Arg
HN
NH2
B
D-Glu
Mdha
L-Leu
Adda
D-Ala
Masp
L-Arg

Fig. 1.15 Microcystin-LR **A.** Linear structure of microcystin-LR. **B.** Solution structure of microcystin-LR. The amino acids are labelled on each structure. Variability in the identity of L-amino acids substituted for L-arginine and/or L-leucine in microcystin-LR accounts for 40 known variants of microcystins, found in both the marine and freshwater environments. Microcystins also possess the unique Adda amino acid in addition to Masp, Glu, and Mdha, as outlined in the text.

molecules). In addition, although both classes of cyclic peptide hepatotoxins are powerful tumour promoters, only nodularins are also tumour initiators. It is believed that the different orientation of the reactive N-methyldehydro-groups of microcystins and nodularins may account for the biochemical differences described above. With PP-1-bound microcystin, the reactive N-methyldehydroalanine side chain is buried in the phosphatase and forms the covalent linkage with Cys-273, while nodularin's reactive N-methyldehydrobutyrine group is oriented in the opposite direction and is probably

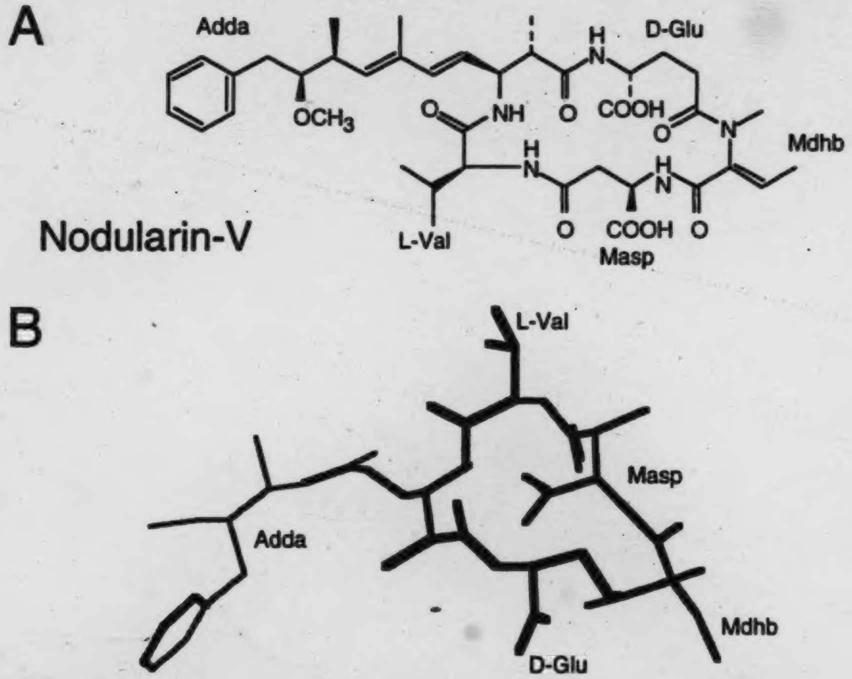


Fig. 1.16 Nodularin-V (motuporin) A. Linear structure of nodularin-V. B. Solution structure of nodularin-V. The amino acids are labelled on each structure. Nodularin-V differs from nodularin-R by substitution of a valine residue for an arginine residue. Nodularins also possess the unique Adda amino acid in addition to Masp, Glu, and Mdhb, as outlined in the text.

exposed to solvent, making it available for interaction with other molecules [190]. Since PP-1c is known to exist in the nucleus, it is possible that nodularin bound to PP-1c could find its way into the nucleus and interact with DNA, leading to the tumour initiator effects of nodularin. With microcystins, the reactive group is buried in the phosphatase and is unable to interact with DNA.

One of the crystal structures of PP-1c determined in 1995 was a heterodimeric complex of PP-1c with microcystin-LR covalently bound to Cys-273 [83]. This structure confirmed the hypothesis that microcystin-LR binds directly in the catalytic center of the phosphatase and raised the question of the molecular mechanism of PP-1c-microcystin-LR inhibitory interactions. Moreover, evidence suggests that okadaic acid binds in the same site as microcystin-LR, since microcystin-LR can compete with okadaic acid for phosphatase binding sites [181,191,192]. However, the precise nature of okadaic acid interactions with PP-1c is not known.

As with okadaic acid, both microcystins and nodularins have little effect upon PP-2B. This raises the question of the molecular basis for PP-2B resistance to these inhibitors in the face of PP-1 sensitivity and such similar catalytic domain protein architecture. Thus several questions remain unanswered regarding the structural basis of toxin interaction with both PP-1 and PP-2B. Chapter 5 of this thesis describes experiments designed to elucidate the molecular mechanism of inhibition of PP-1c by marine toxins and PP-2B resistance to these inhibitors. In this thesis, the crystal structures of both of these phosphatases and structural information for each of the inhibitors are combined to formulate a systematic mutagenesis strategy that is then applied to PP-1c and yields information regarding PP-2B resistance.

1.7 ■ EVOLUTIONARY ASPECTS

Protein Phosphatase-1 is Among the Most Conserved Eukaryotic Enzymes

Of all the known eukaryotic enzymes, PP-1c is among the most conserved throughout evolution [54,55]. I performed a sequence alignment of all the known full-length sequences of PP-1c which demonstrates the high degree of conservation of this enzyme from the lower unicellular eukaryotes through to the higher plants and animals (Fig. 1.17, see also Appendix A). This extreme degree of conservation suggests that PP-1c plays absolutely critical roles in eukaryotic physiology.

Given its high conservation, we are also able to employ the sequences of PP-1c from diverse organisms to construct a tree illustrating the relationships between the sequences. Such a tree provides insight into the phylogenetic position of the organism from which the PP-1c homolog has been sequenced and identifies subgroups which share common sequence themes. Since PP-1c is so highly conserved, sequences that are different between groups may indicate evolutionary divergence. In addition, employing such a phylogenetic tree allows us to trace the relationships between groups and gain insight into the origins of divergent PP-1c sequences.

An analysis of the known PP-1c sequences employing CLUSTALW software [193] was also performed and a phylogenetic tree generated (Fig. 1.18). The most striking feature of this tree is the divergence of the two *Trypanosoma brucei* PP-1c sequences from all other sequences. This is most likely due to a distinctly different sequence present in the amino-terminal of PP-1c from this parasite. This sequence is longer than its counterparts from all the other PP-1c sequences, however, it does contain three sequence motifs that are similar to the other PP-1c sequences. A database search for protein sequences that may contain the unique amino-terminal sequence of *T. brucei*

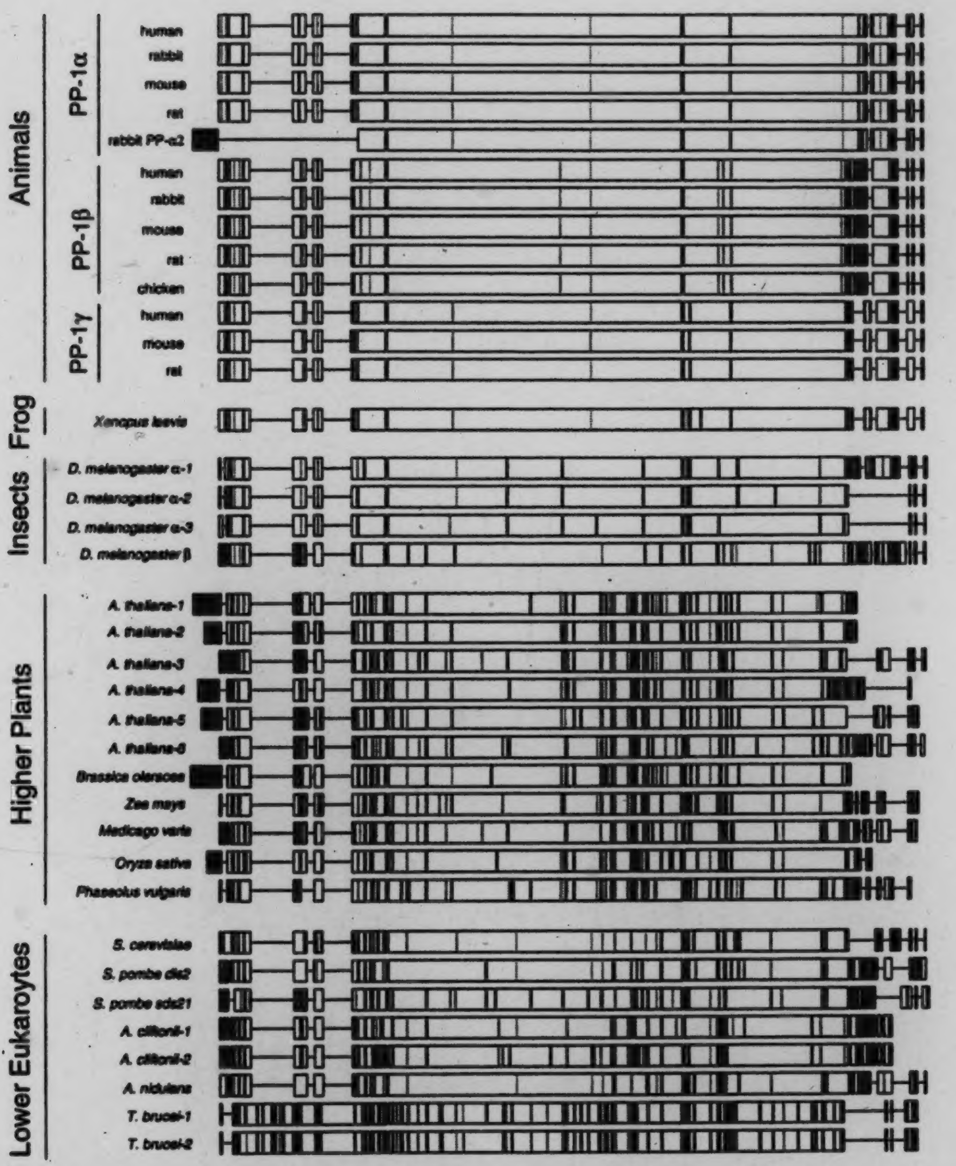

Animals
PP-1α
human
rabbit
mouse
rat
rabbit PP-α2
PP-1β
human
rabbit
mouse
rat
chicken
PP-1γ
human
mouse
rat
Frog
Xenopus laevis
Insects
D. melanogaster α-1
D. melanogaster α-2
D. melanogaster α-3
D. melanogaster β
Higher Plants
A. thaliana-1
A. thaliana-2
A. thaliana-3
A. thaliana-4
A. thaliana-5
A. thaliana-6
Brassica oleracea
Zea mays
Medicago varia
Oryza sativa
Phaseolus vulgaris
Lower Eukaroytes
S. cerevisiae
S. pombe dis2
S. pombe sds21
A. cilliorii-1
A. cilliorii-2
A. nidulans
T. brucei-1
T. brucei-2

Fig. 1.17 Phylogenetic alignment of all known full-length PP-1 primary sequences from diverse eukaryotic organisms. After aligning the sequences, each amino acid position was examined to determine which residue is present in the majority of all the sequences. The amino acid for any given position that represents the majority of sequences is shown in white. Any amino acid that was different from the majority of sequences in that position is shown in black. Any amino acid that was a conserved change in a specific position, e.g. a lysine in a position that is arginine in the majority of the sequences, is shown as a grey line. By inspection, it is noted that the higher plant and animal sequences form two different groups of sequences. Amino acids characteristic of animals tend to also be found among the fungi, while plant-specific amino acids tend to also be present in the green algae.

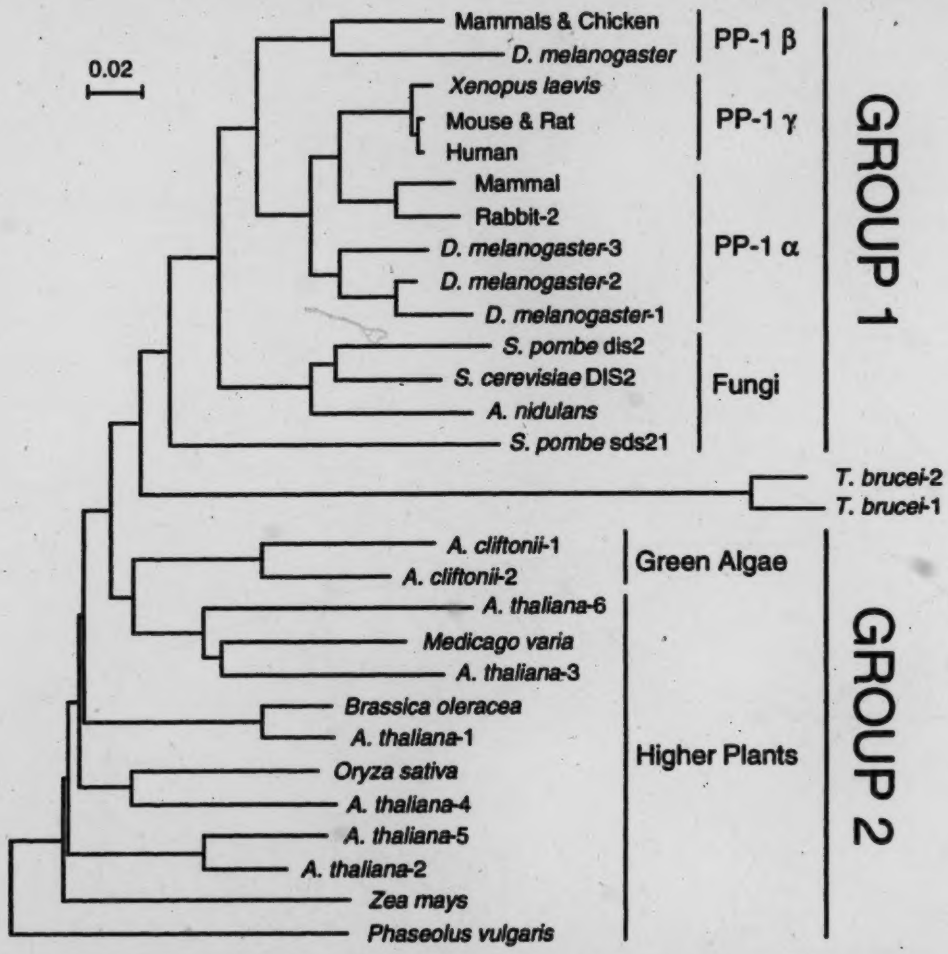

0.02
Mammals & Chicken
D. melanogaster
PP-1 β
Xenopus laevis
Mouse & Rat
Human
PP-1 γ
Mammal
Rabbit-2
D. melanogaster-3
D. melanogaster-2
D. melanogaster-1
PP-1 α
S. pombe dis2
S. cerevisiae DIS2
A. nidulans
Fungi
S. pombe sds21
GROUP 1
T. brucei-2
T. brucei-1
A. cliftonii-1
A. cliftonii-2
Green Algae
A. thaliana-6
Medicago varia
A. thaliana-3
Brassica oleracea
A. thaliana-1
Oryza sativa
A. thaliana-4
A. thaliana-5
A. thaliana-2
Zea mays
Phaseolus vulgaris
Higher Plants
GROUP 2

Fig. 1.18 Phylogenetic tree derived from a comparison of all known full-length sequences of PP-1 from all levels of the eukaryotic radiation. This analysis employed CLUSTALW multiple sequence alignment software. Two main groupings of sequences are apparent: Group 1 consisting of the animals, amphibians, insects, and unicellular fungi and group 2 including the green algae and higher plants. The two PP-1 isoforms from *T. brucei* possess divergent and unique PP-1 sequence motifs which put them in a separate group.

PP-1c returned no significant matches, suggesting that this sequence is either unique or any sequences comparable to it have yet to be identified. It is hypothesized that the amino-terminal sequence of *T. brucei* PP-1c is the result of genetic replacement of the original ancestral PP-1c amino-terminal, which likely contained an amino-terminal more like that of all the other PP-1c sequences, with a piece of DNA that complemented the original DNA in that it possessed the three sequence motifs that appear in PP-1c. These sequences may play important roles for the proper functioning or expression of PP-1c.

Within the remaining PP-1c sequences, two main groups emerge: those of the plants and those of the animals. The formation of these two groups is most likely due to divergent evolution of these forms of life. Significantly, unicellular eukaryotes are found within these two groups, providing insight into the origins of the two more advanced groups. Hence, the PP-1c sequences from yeast and *Aspergillus nidulans* appear to be more related to those of the animals and insects, while PP-1c from green algae is more like plants. This suggests that during evolution plants and animals diverged at an early stage, with the animals and insects originating from the same phylogenetic tree branch as the yeast and fungi. Likewise, the plants evolved separately with the green algae.

Within the animal-track PP-1c sequences, four further subdivisions are readily apparent. First is the group containing the lower eukaryotic PP-1c sequences and then three groups, each corresponding to an isoform of PP-1c (α, β, or γ, see Table 1.3). Within each of these isoform groups, there are additional evolutionary groupings, with the *Drosophila melanogaster* isoforms of PP-1α and PP-1β standing apart from their higher eukaryotic counterparts. Similarly, the frog *Xenopus laevis* PP-1γ is separated from the mammalian forms of this isoform, although these different forms of the γ isoform are remarkably conserved between the amphibian and mammal.

Among the 11 known plant PP-1c sequences, six of which are from *Arabidopsis thaliana*, there are 7 amino acids that are uniformly different from the animal sequences (Asp-11, Ala-85, Val-161, Leu-170, Met-172, Asp-230, and Glu-234), while 19 positions possess a change that is common to at least 8 of the 11 plant sequences. Of the 7 uniformly different amino acid positions, four represent conserved amino acid substitutions. Only one amino acid position possesses a completely different type of amino acid, that being Glu-234 which among the animals is a basic amino acid. The small sequence differences between the plant and animal groups that distinguish these divisions may indicate that these sequences are either not critical for the overall function of PP-1c or that PP-1c plays subtly different roles in these groups that require slight alterations in its sequence. The emergence of the commonly accepted phylogenetic groups [194] from a phylogenetic tree derived from an alignment of all known PP-1c sequences is a testament to the conservation of this enzyme throughout evolution. The striking conservation of this enzyme implies that its functions are indispensable for eukaryotic life forms.

The Phylogenetic Position of Dinoflagellates

My interest in dinoflagellates stems primarily from the fact that some dinoflagellate species are the primary producers of okadaic acid (as discussed in Section 1.6). Dinoflagellates are a very ancient group, being first found in the fossil record about 900 million years ago [195]. Because the fossil record is incomplete, however, it is likely that dinoflagellates are older, perhaps as old as the first eukaryotes which existed approximately 2 billion years ago. The initial observation that dinoflagellate DNA lacked histones and nucleosomes, mitosis occurred through a membranous mechanism, and DNA was replicated continuously fostered the hypothesis that dinoflagellates were intermediate between prokaryotes and eukaryotes [196]. As such, dinoflagellates were initially classified as mesokaryotic to distinguish their intermediate evolutionary nature.

Recently, more detailed evidence is accumulating to suggest that dinoflagellates are indeed true eukaryotes, including the demonstration that mitosis among dinoflagellates involves microtubules and includes a definite cell cycle [197]. RNA sequencing data also suggests that dinoflagellates emerged rather late in eukaryotic evolution in a cluster with the ciliates (Ciliophora) and sporozoans (Apicomplexa) [198].

The phylogenetic position of the dinoflagellates has therefore been the subject of debate during the time that the work described in this thesis was performed. While the work detailed herein deals primarily with the biochemical regulation of PP-1 by marine toxins and reversible protein phosphorylation, the fact that marine dinoflagellates are primary producers of a potent inhibitor of PP-1 has evolutionary significance. The work described herein provides additional evidence pertaining to the correct phylogenetic position of dinoflagellates while aiming to understand the regulation of PP-1 by, among other means, the toxins they produce.

1.8 ■ CHRONOLOGICAL OVERVIEW

The phylogenetic debate concerning the position of dinoflagellates coupled with the biosynthesis of okadaic acid, a potent inhibitor of PP-1, by these primitive unicellular marine organisms serves as the point of departure for the work described in this thesis. A corollary of the mesokaryotic hypothesis was that dinoflagellates may possess signaling molecules that are different from eukaryotes, and thereby account for the ability of *Prorocentrum lima* to exist with high intracellular concentrations of the toxin it produces. Alternatively, if dinoflagellates are eukaryotic, perhaps the signal transduction pathways that regulate its physiological functions are resistant to okadaic acid. In either case, the regulation of signal transduction by okadaic acid in the marine dinoflagellate *P. lima* was of major interest.

Our laboratory therefore undertook to investigate the nature of signal transduction in the okadaic acid-producing marine dinoflagellate *P. lima*. While a postdoctoral fellow set out to isolate and characterize possible PP-1 activity in *P. lima*, I initially examined how protein kinase activity in *P. lima* might be affected by the okadaic acid it produces. I therefore developed a capillary electrophoresis-based assay for protein kinases and protein phosphatases employing peptide substrates to facilitate my work, as outlined in Chapter 2 of this thesis.

It was also of interest to apply the data collected from my biochemical work with dinoflagellates to support or dispute the mesokaryotic hypothesis. In this regard, it was hypothesized that studying signaling molecules in dinoflagellates, and in particular the dinoflagellate that produces okadaic acid, would facilitate a greater understanding of the true phylogenetic character of the dinoflagellates. Thus, Chapter 3 of the thesis outlines the identification and characterization of signaling molecules that employ pTyr or pSer/pThr in marine dinoflagellates with special emphasis on the okadaic acid-producing *P. lima*. The capillary electrophoresis-based protein phosphorylation assay described in Chapter 2 was also applied to this system to identify the presence of PKA in the dinoflagellates.

While our laboratory isolated a sequenceable fragment of DNA encoding PP-1c from *P. lima*, I found that PKA activity in dinoflagellate whole cell extracts seemed to regulate the activity of a PPP family phosphatase. The fragment of the *P. lima* PP-1c homolog was sequenced and contained a classic PKA phosphorylation site. Analysis of all known PP-1c sequences at that time indicated that this putative PKA phosphorylation site was present among several lower eukaryotes. Employing the capillary electrophoresis-based phosphorylation assay, I found that a peptide fragment corresponding to the putative PKA phosphorylation site of *P. lima* PP-1 was a good substrate for PKA *in vitro*. This sparked the hypothesis that PP-1 is regulated directly by PKA phosphorylation among lower eukaryotes. I decided to examine this exciting hypothesis in the fission yeast *S. pombe* because it represents an easily manipulated system for which established biological and genetic protocols are readily available and in which two isoforms of PP-1c are present, one of which contains the putative phosphorylation site (sds21). This work is outlined in Chapter 4 of the thesis.

About the time that my *S. pombe* work was being performed, the crystal structures for PP-1c and PP-2B were published and with them, for the first time, a glimpse of the molecular mechanism of the inhibition of PP-1c by microcystin-LR. A product of this new information was the molecular modeling of okadaic acid and nodularin into the toxin binding pocket of PP-1c by my colleagues in the department. In addition, I was intrigued by the resistance of PP-2B to the marine toxins, despite its possession of strikingly similar active site architecture to PP-1c. Since microcystin-LR bound to the active site of PP-1c, it seemed logical that subtle structural changes in the comparable active site of PP-2B must be responsible for the resistance of this phosphatase to the marine toxins, perhaps by blocking access to the active site. Therefore, we docked the structure of microcystin-LR onto the crystal structure of PP-2B and identified amino acids that conflicted with the structure of the toxin.

Chapter 5 outlines work designed to test the models of marine toxins docked on the crystal structure of PP-1c and to examine the contribution of structural differences between PP-1c and PP-2B on phosphatase sensitivity and resistance to the marine toxins. This work culminates in a hypothesis regarding the molecular mechanism underlying the sensitivity of PP-1 to the marine toxins with an eye to the resistance of PP-2B.

1.9 ■ SPECIFIC AIMS OF THE THESIS

PP-1 is one of only four major classes of protein serine/threonine phosphatases responsible for counterbalancing the activity of hundreds of protein serine/threonine protein kinases, such as cAMP-dependent protein kinase (PKA). PP-1 is therefore a key regulatory enzyme which, together with PP-2A, is responsible for the majority of protein phosphatase activity in eukaryotic cells. PP-1 plays critical roles in a diverse range of physiological processes including glycogen metabolism, regulation of gene expression, and cell cycle control. In addition to its crucial roles in important physiological processes, PP-1 is among the most conserved eukaryotic enzymes throughout evolution, suggesting that it must carry out indispensable functions in eukaryotic cells.

Far from being a constitutively active housekeeping enzyme, PP-1 is actively regulated by a variety of biochemical processes reflecting the diversity of physiological roles which it plays. Intracellular compartmentalization of PP-1 by association with targeting subunits and inhibition of PP-1 activity by inhibitory proteins serve to regulate PP-1 activity in a growing number of physiological processes. In addition, PP-1 activity is regulated directly by phosphorylation of the catalytic subunit by cdc2 kinase, suggesting a central role for PP-1 activity in control of the cell cycle. Finally, PP-1 has been selected by nature as the specific physiological target of a growing number of marine toxins that affect the health of millions of people worldwide, including the causative agent of diarrhetic shellfish poisoning, okadaic acid, and the cyanobacteriallyderived cyclic peptides microcystins and nodularins which threaten the water supplies of people in countries lacking reasonable water purification.

Due the indispensable nature of PP-1 activity, understanding the means by which it is regulated is essential to a full appreciation of the elegant and complex character of PP-1 activity in such an overwhelmingly diverse range of physiological roles. It was therefore the primary objective of this thesis to understand the mechanism by which PP-1 is regulated by reversible protein phosphorylation of the catalytic subunit and by interaction with naturally occurring toxins, specifically the marine toxins. Each chapter contained herein has an objective that relates to the primary goal, more specifically:

1. To develop a capillary electrophoresis assay for protein kinases and protein phosphatases *and thereby* extend our ability to detect and measure the process of reversible protein phosphorylation.

2. To investigate signal transduction involving protein kinases and protein phosphatases among dinoflagellates with particular attention to the okadaic acid-producing species *Prorocentrum lima and thereby* i) provide an understanding of how okadaic acid might play a role in regulating reversible protein phosphorylation-mediated processes in this organism and ii) provide insight into the phylogenetic position of dinoflagellates in general.

3. To test the hypothesis that PP-1 from a significant number of lower eukaryotic organisms is regulated directly by PKA *and thereby* provide insight into the evolution of the regulation of PP-1.

4. To determine the molecular mechanisms underlying the interaction of PP-1 with natural toxins *and thereby* provide an understanding of the molecular mechanism of PP-1 sensitivity and PP-2B resistance to marine toxins.

1.10 ■ BIBLIOGRAPHY

1. Hunter, T. (1995) Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80:** 225-236.

2. Hanks, S. K., Quinn, A.M., and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of catalytic domains. *Science* **241:** 42-52.

3. Hunter, T. (1987) A thousand and one protein kinases. *Cell* **50:** 823-829.

4. Hunter, T., and Cooper, J. A. (1985) Protein-tyrosine kinases. *Annu. Rev. Biochem.* **54:** 897-930.

5. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1990) Oncogenes and signal transduction. *Cell* **64:** 281-302.

6. Aaronson, S. A. (1991) Growth factors and cancer. *Science* **254:** 1146-1153.

7. Taylor, S. S. (1989) cAMP-dependent protein kinase. *J. Biol. Chem.* **264:** 8443-8446.

8. Taylor, S. S., Beuchler, J. A., and Yonemoto, W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* **59:** 971-1005.

9. Taylor, S. S., and Adams, J. A. (1992) Protein kinases: coming of age. *Curr. Opin. Struct. Biol.* **2:** 743-748.

10. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S., and Zoller, M. J. (1993) A template for the protein kinase family. *Trends Biochem. Sci.* **18:** 84-89.

11. Karin, M., and Smeal, T. (1992) Control of transcription factors by signal transduction pathways: the beginning of the end. *Trends Biochem. Sci.* **18:** 418-422.

12. Lalli, E., and Sassone-Corsi, P. (1994) Signal transduction and gene regulation: the nuclear response to cAMP. *J. Biol. Chem.* **269:** 17359-17362.

13. Hunter, T., and Plowman, G. D. (1997) The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.* **22:** 18-22.

14. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253:** 407-414.

15. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253:** 414-420.

16. DeBondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., and Kim, S.-H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature* **363:** 595-602.

17. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994) Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. *Nature* **367:** 704-710.

18. Hu, S.-H., Parker, M. W., Lei, J. Y., Wilce, M. C. J., Benian, G. M., and Kemp, B. E. (1994) Insights into autoregulation from the crystal structure of twitchin kinase. *Nature* **369:** 581-584.

19. Owen, D. J., Noble, M. E., Garman, E. F., Papageorgiou, A. C., and Johnson, L. N. (1995) Two structures of the catalytic subunit of phosphorylase kinase: an active protein kinase complexed with substrate analogue and product. *Structure* **3:** 467-482.

20. Xu, R.-M., Carmel, G., Sweet, R. M., Kuret, J., and Cheng, X. (1995) Crystal structure of casein kinase-1, a phosphate-directed protein kinase. *EMBO J.* **14:** 1015-1023.

21. Longenecker, K. L., Roach, P. J., and Hurley, T. D. (1996) Three-dimensional structure of mammalian casein kinase I: molecular basis for phosphate recognition. *J. Mol. Biol.* **257:** 618-631.

22. Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H., and Huber, R. (1993) Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 Å structure of the complex with Mn^{2+} adenylyl imidodiphosphate and inhibitor peptide PKI(5-24). *EMBO J.* **12:** 849-859.

23. Kemp, B. E., Parker, M. W., Hu, S., Tiganis, T., and House, C. (1994) Substrate and pseudosubstrate interactions with protein kinases: determinants of specificity. *Trends Biochem. Sci.* **19:** 440-443.

24. Kemp, B. E., Benjamini, E., and Krebs, E. G. (1976) Synthetic hexapeptide substrates and inhibitors of 3':5'-cyclic AMP-dependent protein kinase. *Proc. Nat. Acad. Sci. USA* **73:** 1038-1042.

25. Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **252:** 4888-4894.

26. Pearson, R. B., and Kemp, B. E. (1991) Protein kinases phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.* **200:** 62-81.

27. Kemp, B. E., and Pearson, R. B. (1991) Design and use of peptide substrates for protein kinases. *Methods Enzymol.* **200:** 121-134.

28. Tonks, N. K. (1990) Protein phosphatases: key players in the regulation of cell function. *Curr. Opin. Cell Biol.* **2:** 1114-1124.

29. Barford, D. (1995) Protein phosphatases. *Curr. Opin. Struct. Biol.* **5:** 728-734.

30. Shenolikar, S. (1994) Protein serine/threonine phosphatases - new avenues for cell regulation. *Annu. Rev. Cell Biol.* **10:** 55-86.

31. Villafranca, J. E., Kissinger, C. R., and Parge, H. E. (1996) Protein serine/threonine phosphatases. *Curr. Opin. Biotech.* **7:** 397-402.

32. Cohen, P. T. W. (1994) Nomenclature and chromosomal localization of human protein serine/threonine phosphatase genes. *Adv. Prot. Phosphatases* **8:** 371-376.

33. Stone, J. M., Collinge, M. A., Smith, R. D., Horn, M. A., and Walker, J. C. (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. *Science* **266:** 793-795.

34. Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F., and Giraudat, J. (1994) *Arabidopsis* ABA response gene *ABI1*: features of a calcium-modulated protein phosphatase. *Science* **264:** 1448-1452.

35. Meyer, K., Leube, M. P., and Grill, E. (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* **264:** 1452-1455.

36. Maeda, T., Wurgler-Murphy, S. M., and Saito, H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature* **369:** 242-245.

37. Barton, G. J., Cohen, P. T. W., and Barford, D. (1994) Conservation analysis and structure prediction of the protein serine/threonine phosphatases. *Eur. J. Biochem.* **220:** 225-237.

38. Ingebritsen, T. S., and Cohen, P. (1983) Protein phosphatases: properties and role in cellular regulation. *Science* **221:** 331-338.

39. Ingebritsen, T. S., and Cohen, P. (1983) The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. *Eur. J. Biochem.* **132:** 255-261.

40. Perrino, B. A., Ng, L. Y., and Soderling, T. R. (1995) Calcium regulation of calcineurin phosphatase activity by its B subunit and calmodulin. *J. Biol. Chem.* **270:** 340-346.

41. Clipstone, N. A., and Crabtree, G. R. (1992) Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. *Nature* **357:** 695-697.

42. Schreiber, S. L. (1992) Immunophilin-sensitive protein phosphatase action in cell signaling pathways. *Cell* **70:** 365-368.

43. Crabtree, G. R., and Clipstone, N. A. (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem.* **63:** 1045-1083.

44. Loh, C., Shaw, K. T.-Y., Carew, J., Viola, J. P. B., Luo, C., Perrino, B. A., and Rao, A. (1996) Calcineurin binds the transcriptional factor NFAT1 and reversibly regulates its activity. *J. Biol. Chem.* **271:** 10884-10891.

45. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J., and O'Neil, E. A. (1992) FK506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* **357:** 692-694.

46. Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., and Crabtree, G. R. (1989) Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science* **246:** 1617-1620.

47. Lui, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66:** 807-815.

48. Kunz, J., and Hall, M. N. (1993) Cyclosporin, FK506 and rapamycin: more than just immunosuppression. *Trends Biochem. Sci.* **18:** 334-338.

49. Mayer-Jaekel, R. E., and Hemmings, B. A. (1994) Protein phosphatase 2A - a 'ménage a trois'. *Trends Cell Biol.* **4:** 287-291.

50. Ulug, E. T., Cartwright, A. J., and Courtneidge, S. A. (1992) Characterization of the interaction of polyomavirus middle T antigen with type 2A protein phosphatase. *J. Virol.* **66:** 1458-1467.

51. Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993) The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. *Cell* **75:** 887-897.

52. Re, F., Braaten, D., Franke, E. K., and Luban, J. (1995) Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G_2 by inhibiting the activation of $p34^{cdc2}$-cyclin B. *J. Virol.* **69:** 6859-6864.

53. Tung, H. Y. L., De Rocquigny, H., Zhao, L.-J., Cayla, X., Roques, B. P., and Ozon, R. (1997) Direct activation of protein phosphatase-$2A_0$ by HIV-1 encoded protein complex NCp7:vpr. *FEBS Lett.* **401:** 197-201.

54. Cohen, P., and Cohen, P. T. W. (1989) Protein phosphatases come of age. *J. Biol. Chem.* **264:** 21435-21438.

55. Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **59:** 453-508.

56. Bollen, M., and Stalmans, W. (1992) The structure, role, and regulation of type 1 protein phosphatases. *Crit. Rev. Biochem. Mol. Biol.* **27:** 227-281.

57. Mumby, M. C., and Walter, G. (1993) Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. *Physiol. Rev.* **73:** 673-699.

58. Voet, D., and Voet, J. G. (1990) in Biochemistry, pp. 470-479, John Wiley & Sons, Toronto

59. Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990) The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature* **348:** 302-308.

60. Hill, C. S., and Treisman, R. (1995) Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80:** 199-211.

61. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992) Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* **70:** 105-113.

62. Hagiwara, M., Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R., and Montminy, M. R. (1993) Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol. Cell. Biol.* **13:** 4852-4859.

63. Murray, A., and Hunt, T. (1993) in The cell cycle: an introduction, pp. 42-55, Oxford University Press, New York

64. Doonan, J. H., and Morris, N. R. (1989) The *bim*G gene of Aspergillus nidulans, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. *Cell* **57:** 987-996.

65. Booher, R., and Beach, D. (1989) Involvement of a type 1 phosphatase encoded by *bws1*$^+$ in fission yeast mitotic control. *Cell* **57:** 1009-1016.

66. Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T., and Yanagida, M. (1989) The fission yeast *dis2*$^+$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell* **57:** 997-1007.

67. Axton, J. M., Dombradi, V., Cohen, P. T. W., and Glover, D. M. (1990) One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell* **63:** 33-46.

68. Fernandez, A., Brautigan, D. L., and Lamb, N. J. C. (1992) Protein phosphatase type 1 in mammalian cell mitosis: chromosomal localization and involvement in mitotic exit. *J. Cell Biol.* **116:** 1421-1430.

69. Vandré, D. D., and Wills, V. L. (1992) Inhibition of mitosis by okadaic acid: possible involvement of a protein phosphatase 2A in the transition from metaphase to anaphase. *J. Cell Sci.* **101:** 79-91.

70. Walker, D. H., DePaoli-Roach, A. A., and Maller, J. L. (1992) Multiple roles for protein phosphatase 1 in regulating the *Xenopus* early embryonic cell cycle. *Mol. Biol. Cell* **3:** 687-698.

71. Kinoshita, N., Ohkura, H., and Yanagida, M. (1990) Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. *Cell* **63:** 405-415.

72. Clarke, R. R., Hoffman, I., Draetta, G., and Karsenti, E. (1993) Dephosphorylation of cdc25-C by a type-2A protein phosphatase: specific regulation during the cell cycle in *Xenopus* egg extracts. *Mol. Biol. Cell* **4:** 397-411.

73. Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. (1990) Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates *cdc2*/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J.* **9:** 4331-4338.

74. Marshall, C. J. (1991) Tumour suppressor genes. *Cell* **64:** 313-326.

75. Cobrinik, D., Dowdy, S. F., Hinds, P. W., Mittnacht, S., and Weinberg, R. A. (1992) The retinoblastoma protein and the regulation of cell cycling. *Trends Biochem. Sci* **17:** 312-315.

76. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C.-M., and Livingston, D. M. (1989) The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58:** 1085-1095.

77. Goodrich, D. W., Wang, N. P., Qian, Y.-W., Lee, E.-H., and Lee, W.-H. (1991) The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. *Cell* **64:** 293-302.

78. Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look , T., Harlow, E., Dyson, N., and Helin, K. (1993) The retinoblastoma protein binds to a family of E2F transcription factors. *Mol. Cell Biol.* **13:** 7813-7825.

79. Ludlow, J. W., and Nelson, D. A. (1995) Control and activity of type-1 serine/threonine protein phosphatase during the cell cycle. *Sem. Cancer Res.* **6:** 195-202.

80. Ludlow, J. W., Glenening, C. J., Livingston, D. M., and DeCaprio, J. A. (1993) Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol. Cell. Biol.* **13:** 367-372.

81. Alberts, A. S., Thorburn, A. M., Shenolikar, S., Mumby, M. C., and Feramisco, J. R. (1993) Regulation of cell cycle progression and nuclear affinity of the retinoblastoma protein by protein phosphatases. *Proc. Natl. Acad. Sci. USA* **90:** 388-392.

82. Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A. E., Lee, W.-H., and Elledge, S. J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7:** 555-569.

83. Goldberg, J., Huang, H., Kwon, Y., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376:** 745-753.

84. Egloff, M.-P., Cohen, P. T. W., Reinemer, P., and Barford, D. (1995) Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.* **254:** 942-959.

85. Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. T., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995) X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **82:** 507-522.

86. Kissinger, C. R., Parge, H. E., Knighton, D. R., Lewis, C. T., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, R. E., Moomaw, E. W., Gastinel, L. N., Habuka, N., Chen, X., Maldonado, F., Barker, J. E., Bacquet, R., and Villafranca, J. E. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* **378:** 641-644.

87. Sträter, N., Klabunde, T., Tucker, P., Witzel, H., and Krebs, B. (1995) Crystal structure of a purple acid phosphatase containing a dinuclear Fe(III)-Zn(II) active site. *Science* **268:** 1489-1492.

88. Lohse, D. L., Denu, J. M., and Dixon, J. E. (1995) Insights derived from the structure of the Ser/Thr phosphatases calcineurin and protein phosphatase 1. *Structure* **15:** 987-990.

89. Barford, D. (1996) Molecular mechanisms of the protein serine/threonine phosphatases. *Trends Biochem. Sci.* **21:** 407-412.

90. Hubbard, M. J., and Cohen, P. (1991) Targeting subunits for protein phosphatases. *Methods Enzymol.* **201:** 414-427.

91. Hubbard, M. J., and Cohen, P. (1993) On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.* **18:** 172-177.

92. Aitken, A. A., Bilham, T., and Cohen, P. (1982) Complete primary structure of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur. J. Biochem.* **126:** 235-246.

93. Aitken, A., and Cohen, P. (1982) Isolation and characterisation of active fragments of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *FEBS Lett.* **147:** 54-58.

94. Waelkens, E., Goris, J., Di Salvo, J., and Merlevede, W. (1984) Inhibitor-1 phosphatase activity in vascular smooth muscle. *Biochem. Biophys. Res. Comm.* **120:** 397-404.

95. Dawson, J. F., Boland, M. P., and Holmes, C. F. B. (1994) A capillary electrophoresis-based assay for protein kinases and protein phosphatases using peptide substrates. *Anal. Biochem.* **220:** 340-345.

96. MacDougall, L. K., Campbell, D. G., Hubbard, M. J., and Cohen, P. (1989) Partial structure and hormonal regulation of rabbit liver inhibitor-1: distribution of inhibitor-1 and inhibitor-2 in rabbit and rat tissues. *Biochim. Biophys. Acta* **1010:** 218-226.

97. Hemmings, H. C., and Greengard, P. (1984) DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature* **310:** 503-505.

98. Williams, K. R., Hemmings, H. C., LoPresti, M. B., Konigsberg, W. H., and Greengard, P. (1986) DARPP-32, a dopamine and cyclic AMP-regulated neuronal phosphoprotein. *J. Biol. Chem.* **261:** 1890-1903.

99. King, M. M., Huang, C. Y., Chock, P. B., Nairn, A. C., Hemmings, H. C., Chan, K.-F. J., and Greengard, P. (1984) Mammalian brain phosphoproteins as substrates for calcineurin. *J. Biol. Chem.* **259:** 8080-8083.

100. Hemmings, H. C., Nairn, A. C., Elliot, J. I., and Greengard, P. (1990) Synthetic peptide analogs of DARPP-32 (M_r 32,000 dopamine- and cAMP-regulated phosphoprotein), an inhibitor of protein phosphatase-1. *J. Biol. Chem.* **265:** 20369-20376.

101. Holmes, C. F. B., Campbell, D. G., Caudwell, F. B., Aitken, A., and Cohen, P. (1986) Primary structure of inhibitor-2 from rabbit skeletal muscle. *Eur. J. Biochem.* **155:** 173-182.

102. Holmes, C. F. B. (1987) Analysis of the *in vivo* phosphorylation state of protein phosphatase inhibitor-2 from rabbit skeletal muscle by fast-atom bombardment mass spectrometry. *Biochim. Biophys. Acta* **929:** 208-219.

103. Brautigan, D. L., Sunwoo, J., Labbé, J.-C., Fernandez, A., and Lamb, N. J. C. (1990) Cell cycle oscillation of phosphatase inhibitor-2 in rat fibroblasts coincident with $p34^{cdc2}$ restriction. *Nature* **344:** 74-78.

104. Beullens, M., Eynde, A. V., Stalmans, W., and Bollen, M. (1992) The isolation of novel inhibitory polypeptides of protein phosphatase 1 from bovine thymus nuclei. *J. Biol. Chem.* **267:** 16538-16544.

105. Jagiello, I., Beullens, M., Stalmans, W., and Bollen, M. (1995) Subunit structure and regulation of protein phosphatase-1 in rat liver nuclei. *J. Biol. Chem.* **270:** 17257-17263.

106. Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W., and Bollen, M. (1995) Molecular cloning of NIPP-1, a nuclear inhibitor of protein phosphatase-1, reveals homology with polypeptides involved in RNA processing. *J. Biol. Chem.* **270:** 28068-28074.

107. Beullens, M., Eynde, A. V., Bollen, M., and Stalmans, W. (1993) Inactivation of nuclear inhibitory polypeptides of protein phosphatase-1 (NIPP-1) by protein kinase A. *J. Biol. Chem.* **268:** 13172-13177.

108. Jagiello, I., Beullens, M., Vulsteke, V., Wera, S., Sohlberg, B., Stalmans, W., von Gabain, A., and Bollen, M. (1997) NIPP-1, a nuclear inhibitory subunit of protein phosphatase-1, has RNA-binding properties. *J. Biol. Chem.* **272:** 22067-22071.

109. Stralfors, A., Hiraga, A., and Cohen, P. (1985) The protein phosphatases involved in cellular regulation. Purification and characterisation of the glycogen-bound form of protein phosphatase-1 from rabbit skeletal muscle. *Eur. J. Biochem.* **149:** 295-303.

110. Tang, P. M., Bondor, J. A., Swiderek, K. M., and DePaoli-Roach, A. A. (1991) Molecular cloning and expression of the regulatory (R-Gl) subunit of the glycogen-associated protein phosphatase. *J. Biol. Chem.* **266:** 15782-15789.

111. Hubbard, M. J., and Cohen, P. (1989) Regulation of protein phosphatase-1G from rabbit skeletal muscle. *Eur. J. Biochem.* **186:** 701-709.

112. Hubbard, M. J., Dent, P., Smythe, C., and Cohen, P. (1990) Targeting of protein phosphatase 1 to the sarcoplasmic reticulum of rabbit skeletal muscle by a protein that is very similar or identical to the G subunit that directs the enzyme to glycogen. *Eur. J. Biochem.* **189:** 243-249.

113. MacDougall, L. K., Jones, L. R., and Cohen, P. (1991) Identification of the major protein phosphatases in mammalian cardiac muscle which dephosphorylate phospholamban. *Eur. J. Biochem.* **196:** 725-734.

114. Steenaart, N. A. E., Ganim, J. R., Di Salvo, J., and Kranias, E. G. (1992) The phospholamban phosphatase associated with cardiac sarcoplasmic reticulum is a type 1 enzyme. *Arch. Biochem. Biophys.* **293:** 17-24.

115. Wera, S., Bollen, M., and Stalmans, W. (1991) Purification and characterisation of the glycogen-bound protein phosphatase from rat liver. *J. Biol. Chem.* **266:** 339-345.

116. Doherty, M. J., Moorhead, G., Morrice, N., Cohen, P., and Cohen, P. T. W. (1995) Amino acid sequence and expression of the hepatic glycogen-binding (G-L) subunit of protein phosphatase-1. *FEBS Lett.* **375:** 294-298.

117. Alemany, S., and Cohen, P. (1986) Phosphorylase *a* is an allosteric inhibitor of the glycogen and microsomal forms of rat hepatic protein phosphatase-1. *FEBS Lett.* **194:** 194-202.

118. Printen, J. A., Brady, M. J., and Saltiel, A. R. (1997) PTG, a protein phosphatase-1-binding protein with a role in glycogen metabolism. *Science* **275:** 1475-1478.

119. Doherty, M. J., Young, P. R., and Cohen, P. T. W. (1996) Amino acid sequence of a novel protein phosphatase 1 binding protein (R5) which is related to the liver- and muscle-specific glycogen binding subunits of protein phosphatase 1. *FEBS Lett.* **399:** 339-343.

120. Alessi, D., MacDougall, L. K., Sola, M. M., Ikebe, M., and Cohen, P. (1992) The control of protein phosphatase-1 by targeting subunits. *FEBS Lett.* **210:** 1023-1035.

121. Shirazi, A., Iizuka, K., Fadden, P., Mosse, C., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. J. (1994) Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. *J. Biol. Chem.* **269:** 31598-31606.

122. Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J., and Nakano, T. (1994) Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J. Biol. Chem.* **269:** 30407-30411.

123. Chen, Y. H., Chen, M. X., Alessi, D. R., Campbell, D. G., Shanahan, C., Cohen, P., and Cohen, P. T. W. (1994) Molecular cloning of cDNA encoding the 110 kDa and 21 kDa regulatory subunits of smooth muscle protein phosphatase 1M. *FEBS Lett.* **356:** 51-55.

124. Haystead, C. M. M., Gailly, P., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. J. (1995) Molecular cloning and functional expression of a recombinant 72.5 kDa fragment of the 110 kDa regulatory subunit of smooth muscle protein phosphatase 1M. *FEBS Lett.* **377:** 123-127.

125. Gong, M. C., Fuglsang, A., Alessi, D., Kobayashi, S., Cohen, P., Somlyo, A. V., and Somlyo, A. P. (1992) Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium. *J. Biol. Chem.* **267:** 21492-21498.

126. Ohkura, H., and Yanagida, M. (1991) S. pombe *sds22*$^+$ essential for a midmitotic transition encodes a leucine-rich repeat protein that positively modulates protein phosphatase-1. *Cell* **64:** 149-157.

127. Stone, E. M., Yamano, H., Kinoshita, N., and Yanagida, M. (1993) Mitotic regulation of protein phosphatases by the fission yeast sds22 protein. *Curr. Biol.* **3:** 13-26.

128. Dinischiotu, A., Beullens, M., Stalmans, W., and Bollen, M. (1997) Identification of sds22 as an inhibitory subunit of protein phosphatase-1 in rat liver nuclei. *FEBS Lett.* **402:** 141-144.

129. Beullens, M., Stalmans, W., and Bollen, M. (1996) Characterization of a ribosomal inhibitory polypeptide of protein phosphatase-1 from rat liver. *Eur. J. Biochem.* **239:** 183-189.

130. Kenmochi, N., Maeda, N., and Tanaka, T. (1991) The primary structure of chicken ribosomal protein L5. *Biochim. Biophys. Acta* **1088:** 445-447.

131. Hirano, K., Ito, M., and Hartshorne, D. J. (1995) Interaction of the ribosomal protein L5, with protein phosphatase type 1. *J. Biol. Chem.* **270:** 19786-19790.

132. Andres, J. L., Johansen, J. W., and Maller, J. L. (1987) Identification of protein phosphatase 1 and 2B as ribosomal protein S6 phosphatases *in vitro* and *in vivo*. *J. Biol. Chem.* **262:** 14389-14393.

133. Johnson, D. F., Moorhead, G., Caudwell, F. B., Cohen, P., Chen, Y.-H., Chen, M.-X., and Cohen, P. T. W. (1996) Identification of protein phosphatase-1-binding domains on the glycogen and myofibrillar targeting subunits. *Eur. J. Biochem.* **239:** 317-325.

134. Egloff, M.-P., Johnson, D. F., Moorhead, G., Cohen, P. T. W., Cohen, P., and Barford, D. (1997) Structural analysis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16:** 1876-1887.

135. Moorhead, G., MacKintosh, R. W., Morrice, N., Gallagher, T., and MacKintosh, C. (1994) Purification of type 1 protein (serine/threonine) phosphatases by microcystin-sepharose affinity chromatography. *FEBS Lett.* **356:** 46-50.

136. Campos, M., Fadden, P., Alms, G., Qian, Z., and Haystead, T. A. J. (1996) Identification of protein phosphatase-1-binding proteins by microcystin-biotin affinity chromatography. *J. Biol. Chem.* **271:** 28478-28484.

137. Hirano, K., Erdödi, F., Patton, J. G., and Hartshorne, D. J. (1996) Interaction of protein phosphatase type 1 with a splicing factor. *FEBS Lett.* **389:** 191-194.

138. Helps, N. R., Barker, H. M., Elledge, S. J., and Cohen, P. T. W. (1995) Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53. *FEBS Lett.* **377:** 295-300.

139. Patton, J. G., Porro, E. B., Galceran, J., Tempst, P., and Nadal-Ginard, B. (1993) Cloning and characterization of PSF, a novel pre-mRNA splicing factor. *Genes Dev.* **7:** 393-406.

140. Van Eynde, A., Beullens, M., Stalmans, W., and Bollen, M. (1994) Full activation of a nuclear species of protein phosphatase-1 by phosphorylation with protein kinase A and casein kinase-2. *Biochem. J.* **297:** 447-449.

141. Lee, W.-H., Shew, J.-Y., Hong, F. J., Sery, T. W., Donoso, L. A., Young, L.-J., Bookstein, R., and Lee, E. Y.-H. P. (1987) The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature* **329:** 642-645.

142. Brautigan, D. L. (1995) Flicking the switches: phosphorylation of serine/threonine protein phosphatases. *Sem. Cancer Res.* **6:** 211-217.

143. Johansen, J. W., and Ingebritsen, T. S. (1986) Phosphorylation and inactivation of protein phosphatase 1 by pp60$^{v\text{-}src}$. *Proc. Natl. Acad. Sci. USA* **83:** 207-211.

144. Johansen, J. W., and Ingebritsen, T. S. (1987) Effects of phosphorylation of protein phosphatase 1 by pp60$^{v\text{-}src}$ on the interaction of the enzyme with substrates and inhibitors. *Biochim. Biophys. Acta* **928:** 63-75.

145. Belandia, B., Brautigan, D., and Pérez-Martín, J. (1994) Attentuation of ribosomal protein S6 phosphatase activity in chicken embryo fibroblasts transformed by rous sarcoma virus. *Mol. Cell. Biol.* **14:** 200-206.

146. Villa-Moruzzi, E., Zonca, P. D., and Crabb, J. W. (1991) Phosphorylation of the catalytic subunit of type-1 protein phosphatase by the v-abl tyrosine kinase. *FEBS Lett.* **293:** 67-71.

147. Dohadwala, M., da Cruz e Silva, E. F., Hall, F. L., Williams, R. T., Carbonaro-Hall, D. A., Nairn, A. C., Greengard, P., and Berndt, N. (1994) Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases. *Proc. Natl. Acad. Sci. USA* **91:** 6408-6412.

148. Yamano, H., Ishii, K., and Yanagida, M. (1994) Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus sequence and its potential role in cell cycle regulation. *EMBO J.* **13:** 5310-5318.

149. Kwon, Y.-G., Lee, S. Y., Choi, Y., Greengard, P., and Nairn, A. C. (1997) Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase. *Proc. Natl. Acad. Sci. USA* **94:** 2168-2173.

150. Holmes, C. F. B., and Boland, M. P. (1993) Inhibitors of protein phosphatase-1 and -2A; two of the major serine/threonine protein phosphatases involved in cellular regulation. *Curr. Opin. Struct. Biol.* **3:** 934-943.

151. MacKintosh, C., and MacKintosh, R. W. (1994) Inhibitors of protein kinases and phosphatases. *Trends Biochem. Sci.* **19:** 444-448.

152. Sheppeck, J. E., Gauss, C.-M., and Chamberlain, A. R. (1997) Inhibition of the Ser-Thr phosphatases PP1 and PP2A by naturally occurring toxins. *Bioorg. Med. Chem.* **5:** 1739-1750.

153. Yasumoto, T., Oshima, Y., and Yamaguchi, M. (1978) Occurrence of a new type of shellfish poisoning in the Tohoku district. *Bull. Jpn. Soc. Sci. Fish.* **44:** 1249-1255.

154. Yasumoto, T., Oshima, Y., Sugawara, W., Fukuyo, Y., Oguri, H., Igarashi, T., and Fujita, N. (1980) Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. *Bull. Jpn. Soc. Sci. Fish.* **46:** 1405-1411.

155. Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., and Yasumoto, T. (1982) Isolation and elucidation of the causative toxin of the diarrhetic shellfish poisoning. *Bull. Jpn. Soc. Sci. Fish.* **48:** 549-552.

156. Tachibana, K., Scheuer, P. J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y., and Schmitz, F. J. (1981) Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. *J. Am. Chem. Soc.* **103:** 2469-2471.

157. Yasumoto, T., Oshima, Y., Murakami, Y., Nakajima, I., Bagnis, R., and Fukuyo, Y. (1980) Toxicity of benthic dinoflagellates found in coral reef. *Bull. Jpn. Soc. Sci. Fish.* **46:** 327-331.

158. Nakajima, I., Oshima, Y., and Yasumoto, T. (1981) Toxicity of benthic dinoflagellates in Okinawa. *Bull. Jpn. Soc. Sci. Fish.* **47:** 1029-1033.

159. Murakami, Y., Oshima, Y., and Yasumoto, T. (1982) Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. *Bull. Jpn. Soc. Sci. Fish.* **48:** 69-72.

160. Takai, A., Bialojan, C., Troschka, M., and Rüegg, J. C. (1987) Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. *FEBS Lett.* **217:** 81-84.

161. Bialojan, C., and Takai, A. (1988) Inhibitory effects of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256:** 283-290.

162. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. (1988) Okadaic acid: An additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc. Natl. Acad. Sci. USA* **85:** 1768-1771.

163. Herschman, H. R., Lim, R. W., Brankow, D. W., and Fujiki, H. (1989) The tumor promoters 12-O-tetradecanoylphorbol-12-acetate and okadaic acid differ in toxicity, mitogenic activity and induction of gene expression. *Carcinogenesis* **10:** 1495-1494.

164. Nishiwaki, S., Fujiki, H., Suganuma, M., Furuya-Suguri, H., Matsushima, R., Iida, Y., Ojika, M., Yamada, K., Uemura, D., Yasumoto, T., Schmitz, F. J., and Sugimura, T. (1990) Structure-activity relationship within a series of okadaic acid derivatives. *Carcinogenesis* **11:** 1837-1841.

165. Nam, Y. K., Hiro, M., Kimura, S., Fujiki, H., and Imanishi, Y. (1990) Permeability of a non-TPA-type tumor promoter, okadaic acid, through lipid bilayer membrane. *Carcinogenesis* **11:** 1171-1174.

166. Cohen, P., Holmes, C. F. B., and Tsukitani, Y. (1990) Okadaic acid: a new probe for studying cellular regulation. *Trends Biochem. Sci.* **15:** 98-102.

167. Galey, F. D., Beasly, V. R., Carmichael, W. W., Kleppe, G., Hooser, S. B., and Haschek, W. M. (1987) Blue-green algae (*Microcystis aeruginosa*) hepatotoxicosis in dairy cows. *Am. J. Vet. Res.* **48:** 1415-1420.

168. Francis, G. (1878) Poisonous Australian lake. *Nature* **18:** 11-12.

169. Carmichael, W. W. (1994) The toxins of cyanobacteria. *Sci. Am.* **270:** 78-86.

170. Lambert, T. W., Holmes, C. F. B., and Hrudey, S. E. (1994) Microcystin class of toxins: health effects and safety of drinking water supplies. *Environ. Rev.* **2:** 167-186.

171. Jochimsen, E. M., Carmichael, W. W., An, J., Cardo, D. M., Cookson, S. T., Holmes, C. E. M., de C. Antunes, B., de Melo Filho, D. A., Lyra, T. M., Barreto, V. S. T., Azevedo, S. M. F. O., Jarvis, W. R. (1998) Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N. Engl. J. Med.* **338:** 873-878.

172. Botes, D. P., Tuinman, A. A., Wessels, P. L., Viljoen, C. C., Kruger, H., Williams, D. H., Santikarn, S., Smith, R. J., and Hammond, S. J. (1984) The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*. *J. Chem. Soc. Perkin Trans.* **1:** 2311-2318.

173. Eriksson, J. E., Paatero, G. I. L., Meriluoto, J. A. O., Codd, G. A., Kass, G. E. N., Nicotera, P., and Orrenius, S. (1989) Rapid microfilament reorganization induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. *Exp. Cell Res.* **185:** 86-100.

174. Eriksson, J. E., Toivola, D., Meriluoto, J. A. O., Karaki, H., Han, Y.-G., and Hartshorne, D. (1990) Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. *Biochem. Biophys. Res. Comm.* **173:** 1347-1353.

175. Matsushima, R. N., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W. W., and Fujiki, H. (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J. Cancer Res. Clin. Oncol.* **118:** 420-424.

176. Nishiwaki-Matsushima, R., Nishiwaka, S., Ohta, T., Yoshizawa, S., Suganuma, M., Harada, K., Watanabe, M. F., and Fujiki, H. (1992) Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatases. *Jpn. J. Cancer Res.* **82:** 993-996.

177. Ohta, T., Sueoka, E., Iida, N., Komori, A., Suganuma, M., Nishiwaki, R., Tatematsu, M., Kim, S.-J., Carmichael, W. W., and Fujiki, H. (1994) Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Res.* **54:** 6402-6406.

178. Fujiki, H., and Suganuma, M. (1996) Naturally-derived tumor promoters and inhibitors of carcinogenesis. *J. Toxicol.* **15:** 129-156.

179. Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L., Khatra, B. S., Dukelow, M., and Boynton, A. L. (1990) Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* **265:** 19401-19404.

180. Yoshizawa, S., Matsushima, R., Watanabe, M. F., Harada, K. I., Ichihara, K., Carmichael, W. W., and Fujiki, H. (1990) Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. *J. Cancer Res. Clin. Oncol.* **116:** 609-614.

181. MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P., and Codd, G. A. (1990) Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* **264:** 187-192.

182. Rinehart, K. L., Harada, K. I., Namikoshi, M., Chen, C., Harvis, C. A., Munro, M. H. G., Blunt, J. W., Mulligan, P. E., Beasley, V. R., and Dahlem , A. M. (1988) Nodularin, microcystin and the configuration of Adda. *J. Am. Chem. Soc.* **110:** 8557-8558.

183. Craig, M., McCready, T. L., Luu, H. A., Smillie, M. A., Dubord, P., and Holmes, C. F. B. (1993) Identification and characterization of hydrophobic microcystins in Canadian freshwater cyanobacteria. *Toxicon* **31:** 1541-1549.

184. de Silva, E. D., Williams, D. E., Andersen, R. J., Klix, H., Holmes, C. F. B., and Allen, T. M. (1992) Motuporin, a potent protein phosphatase inhibitor isolated from the Papua New Guinea sponge *Theonella swinhoei* Gray. *Tetra. Lett.* **33:** 1561-1564.

185. Chen, D. Z., Boland, M. P., Smillie, M. A., Klix, H., Ptak, C., Andersen, R. J., and Holmes, C. F. B. (1993) Identification of protein phosphatase inhibitors of the microcystin class in the marine environment. *Toxicon* **31:** 1407-1414.

186. Andersen, R. J., Luu, H. A., Chen, D. Z. X., Holmes, C. F. B., Kent, M. L., Le Blanc, M., Taylor, F. J. R., and Williams, D. E. (1993) Chemical and biological evidence links microcystins to salmon 'netpen liver disease'. *Toxicon* **31:** 1315-1323.

187. Williams, D. E., McCready, T. L., Craig, M., Dawe, S. C., Kent, M. L., Holmes, C. F. B., and Andersen, R. J. (1997) Evidence for a covalently bound form of microcystin-LR in salmon liver and dungeness crab larvae. *Chem. Res. Toxicol.* **10:** 463-469.

188. MacKintosh, R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T. W., Cohen, P., and MacKintosh, C. (1995) The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Lett.* **371:** 236-240.

189. Craig, M., Luu, H. A., McCready, T. L., Williams, D. E., Andersen, R. J., and Holmes, C. F. B. (1996) Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. *Biochem. Cell. Biol.* **74:** 569-578.

190. Bagu, J. R., Sykes, B. D., Craig, M. M., and Holmes, C. F. B. (1997) A molecular basis for different interactions of marine toxins with protein phosphatase-1. *J. Biol. Chem.* **272:** 5087-5097.

191. Matsushima, R., Yoshizawa, S., Watanabe, M. F., Harada, K., Mitsuru, F., Carmichael, W. W., and Fujiki, H. (1990) *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin fibroblasts. *Biochem. Biophys. Res. Comm.* **171:** 867-874.

192. Takai, A., Sasaki, K., Nagai, H., Mieskes, G., Isobe, M., Isono, K., and Yasumoto, T. (1995) Inhibition of specific binding of okadaic acid to protein phosphatase 2A by microcystin-LR, calyculin-A and tautomycin: method of analysis of interactions of tight-binding ligands with target protein. *Biochem. J.* **306:** 657-665.

193. Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.* **266:** 383-402.

194. Woese, C. R. (1996) Phylogenetic trees: whither microbiology? *Curr. Biol.* **6:** 1062-1063.

195. Bujak, J. P., and Williams, G. L. (1981) The evolution of dinoflagellates. *Can. J. Bot.* **59:** 2077-2087.

196. Rizzo, P. J. (1991) The enigma of the dinoflagellate chromosome. *J. Protozool.* **38:** 246-252.

197. Raikov, I. B. (1995) The dinoflagellate nucleus and chromosomes: Mesokaryote concept reconsidered. *Acta Protozool.* **34:** 239-247.

198. Cavalier-Smith, T. (1993) Kingdom protozoa and its 18 phyla. *Microbiol. Revs.* **57:** 953-994.

Chapter 2

Analysis of Protein Phosphorylation by Capillary Electrophoresis*

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* A version of this chapter has been published.

Dawson, J. F., Boland, M. P., and Holmes, C. F. B. (1994) A Capillary Electrophoresis-Based Assay for Protein Kinases and Protein Phosphatases Using Peptide Substrates. *Anal. Biochem.* **220:** 340-345.

2.1 ■ INTRODUCTION

Reversible protein phosphorylation on target serine, threonine, or tyrosine residues by protein kinases and protein phosphatases is a principal mechanism by which extracellular signals are transduced inside eukaryotic cells [1]. Identification of physiological processes which are governed by reversible protein phosphorylation has been facilitated by delineating the roles of specific protein kinases and phosphatases. Therefore, detection of these activities in cell extracts is a necessary step in the elucidation of signal transduction pathways.

As discussed in section 1.5, the activity of protein phosphatase-1 (PP-1), one of the major protein serine/threonine phosphatases responsible for regulating a range of important physiological processes, is regulated by reversible protein phosphorylation. The paradigm for such regulation is the interaction of PP-1 with the glycogen-binding subunit (G-subunit) which targets PP-1 to glycogen particles where the phosphatase interacts with glycogen-bound substrates, discussed in section 1.4. Phosphorylation of the G subunit when it is associated with the PP-1 catalytic subunit, referred to as the PP-1G complex, on two different sites results in opposite effects on PP-1 glycogen-associated activity. Phosphorylation by cAMP-dependent protein kinase (PKA) results in dissociation of the PP-1G complex by introducing a disruptive phosphate into the region of the G-subunit that binds the PP-1 catalytic subunit. Alternatively, phosphorylation of the G-subunit by insulin-stimulated protein kinase (ISPK) increases the activity of PP-1G toward glycogen-bound substrates.

One of the glycogen-associated substrates of PP-1, glycogen synthase, is also regulated by phosphorylation on multiple sites. Phosphorylation of glycogen synthase generally leads to decreased activity of the enzyme. One such phosphorylation event is catalyzed by PKA, as discussed in section 1.3. Conversely, phosphorylation of glycogen synthase by casein kinase-II (CK-II) has little effect on enzymatic activity [2]. However, phosphorylation of glycogen synthase by CK-II provides a phosphoserine residue that is part of the phosphorylation consensus sequence recognized by glycogen synthase kinase-3 (GSK-3), which phosphorylates a serine residue four amino acids C-terminal to a phosphoserine [3] (see Table 1.1). Phosphorylation of glycogen synthase on four serine residues by GSK-3 causes a significant reduction in its activity [4].

As such, phosphorylation of a protein on one site can influence the phosphorylation of another site. Once an initiator phosphoserine has been formed, it has been hypothesized that GSK-3 is able to phosphorylate a substrate on multiple sites processively, since it is able to create its own consensus sequence by providing a phosphoserine for the next consensus sequence in a series. This kind of ordered multisite phosphorylation has been termed hierarchical phosphorylation [3,5]. Phosphorylation of glycogen synthase by GSK-3 on four evenly spaced serine residues following initial phosphorylation by CK-II is believed to occur by hierarchical phosphorylation since it is thought that each phosphorylation depends on the previous phosphorylation event.

The objective of the work described in this chapter was to develop a capillary electrophoresis-based technique to extend our ability to detect and measure the process of reversible protein phosphorylation. Since phosphorylation is intimately involved in the regulation and function of PP-1, it was important to study phosphorylation of substrates and phosphorylation by kinases that are relevant to PP-1. As such, PKA phosphorylation was chosen to develop the assay described herein since PKA activity is often antagonistic to PP-1 activity in physiological processes such as glycogen metabolism and the regulation of gene expression, as outlined in section 1.3. Similarly, it was of interest to analyze the phosphorylation of the G subunit of PP-1G by PKA and CK-II to examine the ability of capillary electrophoresis to analyze multisite phosphorylation. In addition, I employed the assay outlined here to analyze the hypothesis that glycogen synthase is subject to hierarchical phosphorylation by GSK-3 following phosphorylation by CK-II. The assay proved to be successful for the analysis of multisite and hierarchical phosphorylation and, as we shall see, the technique outlined herein was employed in other work described in this thesis to investigate novel systems and targets of reversible protein phosphorylation.

Protein Kinase Peptide Substrates

It is generally accepted that protein kinases recognize local structure surrounding the phosphoacceptor group on their target substrates and that sites phosphorylated by specific protein kinases share a common set of sequence elements which is necessary and sufficient for recognition and phosphorylation by that kinase, as discussed in section 1.1. This set of sequence elements is referred to as a consensus sequence [6]. Consensus sequences are generally short peptide sequences that constitute the minimum set of amino acids required for phosphorylation of a phosphoacceptor residue (see Table 1.1).

Consensus sequences have been employed to synthesize model peptides for the study of protein kinase substrate specificity and to measure protein kinase activity in cell extracts [7,8]. The specificity and recognition sites of PKA have been extensively studied employing peptide substrates [9-12]. These studies determined that PKA recognizes and phosphorylates the consensus sequence Arg-Arg-Xaa-Ser. A small fragment of pyruvate kinase known as Kemptide (Table 2.1) contains this consensus sequence and is commonly used for the detection of PKA activity.

Fragments of known *in vivo* protein kinase substrates have also been used to detect protein kinase activity in cell extracts. For example, a 14-amino acid peptide corresponding to part of the G-subunit of protein phosphatase-1 (referred to as PP-1G peptide) is phosphorylated on Ser-9 by PKA [13]. This peptide is also phosphorylated on Ser-11 by casein kinase-II (CK-II), which recognizes the consensus sequence Ser-Xaa-Xaa-Glu [14]. Similarly, a key regulatory region of glycogen synthase (termed the GS peptide) is phosphorylated *in vivo* by CK-II on Ser-21 and by glycogen synthase kinase-3 (GSK-3) on Ser-5, 9, 13 and 17 [3,5].

Table 2.1 Peptide fragments of *in vivo* protein kinase substrates used to detect protein kinase activity. Phosphorylation sites are indicated in bold. Consensus sequences for PKA (RRXS) and casein kinase-II (SXXE) are underlined with their phosphoacceptor sites in bold. Sites phosphorylated by GSK-3 on the GS peptide are indicated in bold. See Table 1.1 for a list of selected protein kinase consensus sequences.

Name	Sequence	Acting Kinases
Kemptide	LRRA**S**LG	PKA
PP-1G	SPQPSRRG**S**E**S**SEE	PKA, CK-II
GS	PRPA**S**VPP**S**PSL**S**RHS**S**PHQ**S**EDEEEP	CK-II, GSK-3

Assay of protein kinase activity in cell extracts employing peptides possessing protein kinase phosphorylation consensus sequences typically involves monitoring the transfer of a ^{32}P-containing γ-phosphate from ATP to the peptide [15,16]. This requires special handling, washing procedures and shielding to minimize exposure to radiation. Alternatively, synthetic peptides containing a phosphorylation consensus sequence have been engineered to also possess an amino acid residue that demonstrates altered spectrophotometric qualities upon phosphorylation of the peptide [17]. The engineered amino acid residue normally lies close to the phosphorylation site to achieve optimal measurement of phosphorylation. As a result, the kinase consensus sequence is altered, lowering the sensitivity of the assay. A technique that is non-radioactive and employs optimal kinase consensus sequences would be a viable alternative.

Capillary Electrophoresis

The separation of biomolecules in complex mixtures is a crucial step in the analysis and understanding of multi-component biological systems. Biochemical research has benefited from the application of electrophoresis, the separation of charged biomolecules in an applied electrical field, since it was first demonstrated by Tiselius in 1937 [18].

Presently electrophoresis is an indispensable tool in biochemistry, employed in the analysis of all fundamental biological molecules.

The separation of molecules by electrophoresis in capillaries was first described by Hjerten in 1967 [19]. With capillary electrophoresis, a sample is introduced into a capillary that is equilibrated with a single continuous buffer [20-23]. An electrical field is then applied to the system resulting in electrophoretic separation of compounds which pass by a detector.

There are several advantages to carrying out electrophoresis in capillaries. First, internal diameters ranging from 20-100 µm yield high surface-to-volume ratios, resulting in efficient dissipation of heat generated by the application of electrical fields. This permits the use of high electrical fields (up to 30 kV) and reduces separation time compared to other electrophoretic methods. Second, very small sample volumes can be analyzed. A typical analysis can be performed with a sample volume of 5 µL where as little as 0.2 nl is actually analyzed in a single run. Finally, all molecules are detected at the cathode due to the net movement of the buffer in that direction. This phenomenon is known as electroosmotic flow (EOF). When using fused-silica capillaries, bare silica groups impart a negative charge to the inner surface of the capillary, inducing the adsorption of anions at the capillary surface. This produces an excess of cations in the bulk solution inside the capillary, resulting in the net movement of the buffer toward the cathode. EOF is strong enough to cause the movement of all molecules, regardless of their charge, toward the cathode and past the detector. Thus, separation observed in capillaries is the result of both the bulk movement of the solution and the electrophoretic mobility of the compounds in the sample.

Despite these advantages, capillary electrophoresis has not been widely exploited as an analytical tool until recently. Computer technology now combines rapid automated analysis with high resolution and the quantitative precision and control of high performance liquid chromatography (HPLC). However, it has been recent advances in detection systems that has really set capillary electrophoresis apart from other analytical techniques. Employing standard ultraviolet (UV) optics, picomole to femtomole (10^{-12} to 10^{-15} mol) amounts are readily detectable. However, in combination with laser-induced fluorescence (LIF) detection systems, capillary electrophoresis is capable of detecting attomole to zeptomole (10^{-18} to 10^{-21} mol) quantities. Most recent capillary electrophoresis applications have been developed to detect very small amounts of peptides or nucleic acids by LIF [24,25].

The objective of work outlined in this chapter was to combine the analytical power of capillary electrophoresis with our understanding of protein kinase phosphorylation of peptide fragments of *in vivo* substrates related to PP-1 activity to develop a non-radioactive method of protein kinase detection and assay. Ultimately, capillary electrophoresis was employed to reproducibly resolve phosphorylated and unphosphorylated protein kinase peptide substrates. Utilizing Kemptide as the peptide substrate, PKA activity, which often opposes PP-1 activity in the regulation of physiological processes, was monitored as a function of the formation of phosphorylated peptide as seen by capillary electrophoresis analysis. Kemptide was also employed to detect PKA activity in cell extracts using capillary electrophoresis.

Capillary electrophoresis was also utilized to analyze the phosphorylation of PP-1G peptide by PKA and CK-II. Not only was capillary electrophoresis capable of resolving different degrees of phosphorylation (i.e., unphosphorylated versus phosphorylated on one site versus phosphorylated on multiple sites), but the capillary electrophoresis protocol described in this chapter was capable of resolving peptides that have been phosphorylated once on different sites. This procedure may therefore be applied to direct, nonradioactive assay of multisite protein kinase activity, such as that observed on the G-subunit of PP-1G. To test this, the ability of a fragment of glycogen synthase to be processively phosphorylated on multiple sites by GSK-3 was examined. It was demonstrated that for each degree of phosphorylation only one peak was present, supporting the hypothesis that glycogen synthase is subject to hierarchical phosphorylation by GSK-3. Finally, the increased sensitivity of LIF detection of phosphorylated peptides coupled to capillary electrophoresis separation was demonstrated.

2.2 ■ MATERIALS AND METHODS

Reagents and Materials

ATP, KCN, cAMP, cAMP-dependent protein kinase, and Kemptide were obtained from Sigma. Protease inhibitors and casein kinase-II were from Boehringer-Mannheim. 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) was from Beckman.

Preparation of PP-1G Peptide

PP-1G and GS peptides were synthesized on an Applied Biosystems 430A synthesizer using solid-phase methodology and standard protocols incorporating *t*-butoxycarbonyl amino acids. Amino acid coupling was mediated by dicyclohexylcarbodiimide activation and deprotection was carried out with 50% trifluoroacetic acid (TFA) in methylene chloride. Side-chain protecting groups and resin were removed by treatment with liquid HF containing anisole and 10% dimethyl sulfoxide (v/v) at -5 °C for 60 min. Peptides were purified by reverse-phase liquid chromatography (RPLC) on a Vydac C_{18} 218TP54 column (4.6 x 250 mm) using Hewlett-Packard HP1090M instrumentation. Peptides were eluted by linear gradients of acetonitrile (0.1% TFA) in water (0.1% TFA) and were >95% pure as judged by RPLC, amino acid analysis, and mass spectrometry.

Preparation of Rabbit Muscle Extracts for Kinase Assays

Muscle extracts were prepared as outlined by previous workers [26]. For protein kinase assays, the tissue extract was adjusted to pH 7.2 with ammonium hydroxide, and ammonium sulfate was added to a final saturation of 55%. After standing at 4 °C for 30 min, the supernatant was decanted and spun at 4400 *g* for an additional 40 min. The precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.5, and protease inhibitors were added as described. The extract was concentrated in a Centricon-10 filter (Amicon) at 3000 *g* until 20% of the original volume was obtained.

Protein Kinase Assays

Crude muscle extract (20 µL) or the ammonium sulfate cut was incubated with 5 µL of 25 µM cAMP for 15 min at 30 °C. The kinase reaction was started by the addition of 35 µL of a reaction mixture comprising 56 mM Tris-HCl, pH 7.5, 25 mM magnesium acetate, 0.56 mM ATP, and 0.175 mM Kemptide. Reactions were quenched by the addition of an equal volume of 150 mM H_3PO_4, pH 2.0. Samples were then centrifuged at 16000 *g* for 10 min at 4 °C and an aliquot (5-10 µL) of the resulting supernatant was analyzed by capillary electrophoresis. Alternatively, a solution (25 µL) comprising purified PKA catalytic subunit (250 Units reconstituted in 250 µL water containing 6 mg/mL dithiothreitol) was added to the protein kinase reaction mixture (35 µL) and incubated at 30 °C for 10 min. Aliquots (5 µL) were removed at timed intervals and analyzed by capillary electrophoresis.

To analyze multiple phosphorylation sites, PP-1G peptide was used as substrate in kinase assays where a mixture (35 µL) comprising 56 mM Tris-HCl, pH 7.5, 25 mM magnesium acetate, 130 mM potassium chloride, 0.56 mM ATP, and 0.175 mM PP-1G peptide was added to a solution (25 µL) of 50 mM Tris-HCl, pH 7.5, and 0.1% β-mercaptoethanol containing 25 Units PKA catalytic subunit and/or 2 milliunits CK-II. The phosphorylation mixture was incubated at 30 °C and samples (5 µL) were removed and quenched at timed intervals prior to analysis by capillary electrophoresis

To analyze the hypothesis that glycogen synthase is subject to hierarchical phosphorylation, GS peptide was employed in kinase assays which included glycogen synthase kinase-3 (GSK-3) and/or CK-II under the conditions described above for the phosphorylation of PP-1G peptide.

Capillary Electrophoresis

All capillary electrophoresis analyses were performed on a Beckman PACE System 2100 using an uncoated fused-silica capillary of 50 μm internal diameter and 50 cm effective length. Analyses were carried out in 150 mM H_3PO_4 buffer (pH 2.0), except when protein kinase assays utilizing PP-1G peptides as substrate were performed, in which case analyses were performed in 150 mM H_3PO_4 buffer at pH 5.0. Separations were achieved at applied constant voltage (20 kV) for 20 min at 25 °C. Sample injections (pressure mode) were 30 nl and detection was at 200 nm.

Laser-Induced Fluorescence Capillary Electrophoresis

A sample of peptide standard (pH 8.5) was mixed in a fume hood with 20 mM KCN and 10 mM CBQCA (in methanol) in a 1:2:1 volumetric ratio, vortexed for 30 sec and incubated at room temperature for 2 hours.

Analysis of derivatized peptide was performed on a Beckman PACE 2100 System coupled to a PACE LIF Detector Module employing an argon ion laser emitting at 488 nm. Access to a laser-induced detection system for capillary electrophoresis was provided by Dr. E. Leblanc, Department of Home Economics, University of Alberta. Samples were injected (pressure mode) onto a pre-treated fused-silica capillary of 75 μm internal diameter and 50 cm effective length housed in a Beckman LIF Capillary Cartridge. Analyses were carried out in borate buffer (pH 8.3) at applied constant voltage (20 kV) for 20 min at 25 °C.

2.3 ■ RESULTS

Assay of cAMP-dependent Protein Kinase Activity Employing Kemptide as Substrate by Capillary Electrophoresis

Phosphorylation of Kemptide by purified PKA was monitored by capillary electrophoresis as described in the legend to Fig. 2.1. At time zero (Fig. 2.1A), only unphosphorylated peptide was detected in the reaction mixture, seen as a reproducible single peak with a retention time of approximately ten minutes. As the reaction progressed, phosphorylated peptide was produced and seen clearly as a peak eluting at a time of approximately 14 minutes. Both unphosphorylated and phosphorylated peaks were verified by adding a defined amount of appropriate standard to the analyses to enhance the elution position of the unknown peak, thereby facilitating its identification. Herein, this method of peak identification will be referred to as "spiking". These results demonstrated the potential of capillary electrophoresis to quantitate the stoichiometry of phosphorylation of protein kinase substrate peptides (Fig. 2.1 inset).

Detection of cAMP-dependent Protein Kinase Activity Using Capillary Electrophoresis

Initially, the ability of capillary electrophoresis to detect PKA activity in tissue extracts was determined. The ability of Kemptide to be phosphorylated in ammonium sulfate-treated rabbit skeletal muscle extracts was examined (Fig. 2.2). In control experiments where no ATP was added to the kinase reaction mixture (Fig. 2.2C), a peak corresponding to unphosphorylated Kemptide (Fig. 2.2A) was clearly detected over and above compounds in the extract alone (Fig. 2.2B). In the presence of ATP, phosphorylated Kemptide (Fig. 2.2D) was resolved from components of the extract (Fig. 2.2E) and verified by spiking the sample with phosphorylated Kemptide (Fig. 2.2F).

Phosphorylated Kemptide could also be detected in crude muscle extracts using similar procedures (Fig. 2.3). In this case, the amount of background absorbance from the crude extract was significant. However, both unphosphorylated and phosphorylated Kemptide peaks were resolved from the components of the extract and the presence of Kemptide phosphorylated by PKA in the extract was verified by spiking the sample with phosphorylated Kemptide. These results demonstrated the ability of capillary electrophoresis to detect phosphopeptides in complex mixtures such as tissue extracts.

Resolution by Capillary Electrophoresis of a Peptide Phosphorylated on Different Sites

I wished to test whether the capillary electrophoresis approach described above could be applied to the analysis of multisite phosphorylation of a single substrate. Since PP-1 activity is regulated by the phosphorylation of the G-subunit of PP-1G on multiple sites, it was of interest to analyze multisite phosphorylation employing a fragment of the G-subunit. This fragment, referred to as the PP-1G peptide, contains two phosphorylation sites: one phosphorylated by PKA and another phosphorylated by CK-II. Phosphorylation of PP-1G peptide by PKA or CK-II alone was monitored by capillary

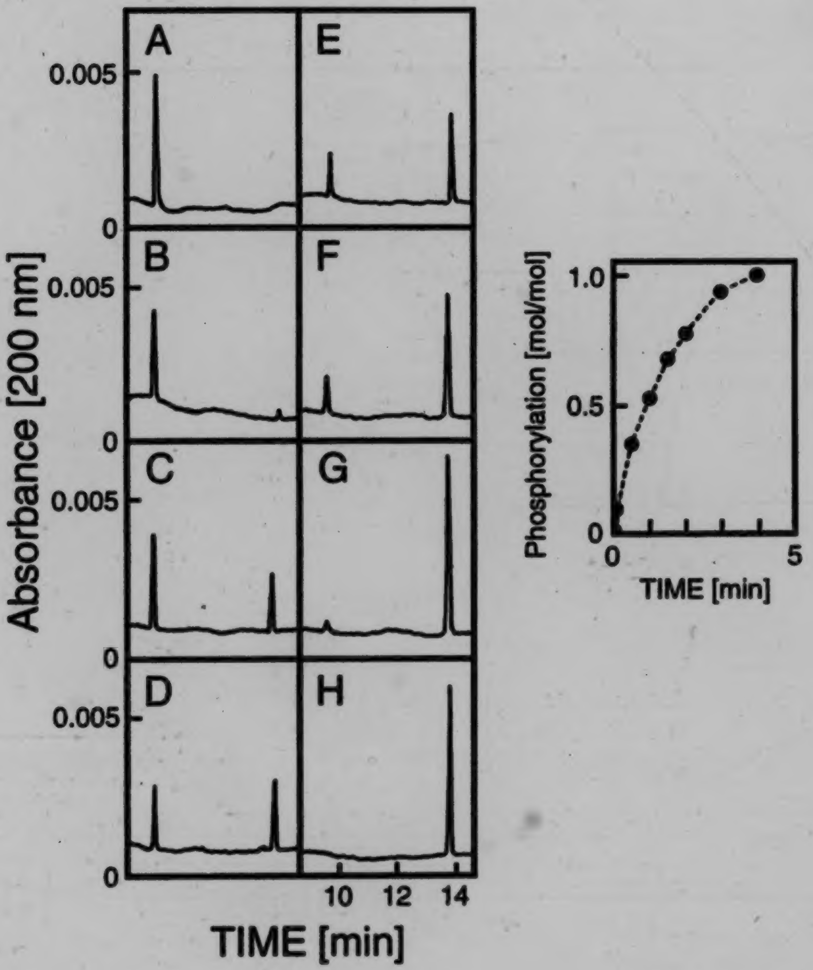


Fig. 2.1 Analysis of the phosphorylation of Kemptide by purified cAMP-dependent protein kinase using capillary electrophoresis. Time points of analysis were 0 min (A), 0.1 min (B), 0.5 min (C), 1.0 min (D), 1.5 min (E), 2.0 min (F), 3.0 min (G), and 4.0 min (H). Capillary electrophoresis was performed as described in Materials and Methods and the total amount of Kemptide applied to the capillary was 2.6 pmol in each case. The phosphorylation reaction was completed at 4.0 min. A plot of the rate of phosphorylation of Kemptide by PKA was produced (inset) by calculating the percentage of the total peak areas of dephosphopeptide and phosphopeptide at each time point.

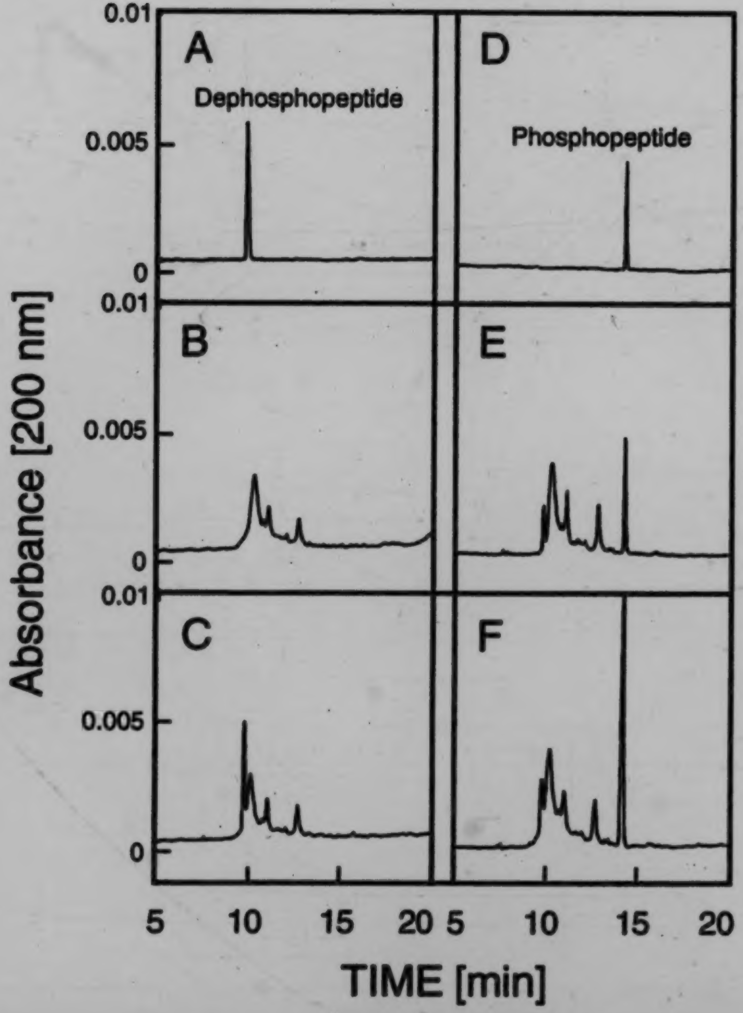


Fig. 2.2 Detection of cAMP-dependent protein kinase activity in ammonium sulfate-treated rabbit skeletal muscle extracts employing capillary electrophoresis. A. Elution position of standard dephosphorylated Kemptide. B. Ammonium sulfate-treated extract alone. C. Ammonium sulfate-treated extract incubated with unphosphorylated Kemptide in the absence of exogenous ATP. No significant phosphorylation of Kemptide is observed. D. Standard phosphorylated Kemptide elution position. E. Extract incubated with dephosphorylated Kemptide in the presence of exogenous ATP. Note the emergence of a sharp peak with an elution time corresponding to that of phosphorylated Kemptide standard shown in D. F. Verification of the presence of phosphopeptide in the reaction described in E by spiking the sample with phosphopeptide standard. The increase in the peak confirms its identity as phosphorylated Kemptide. In C and E, the total amount of Kemptide applied to the capillary was 2.6 pmol.

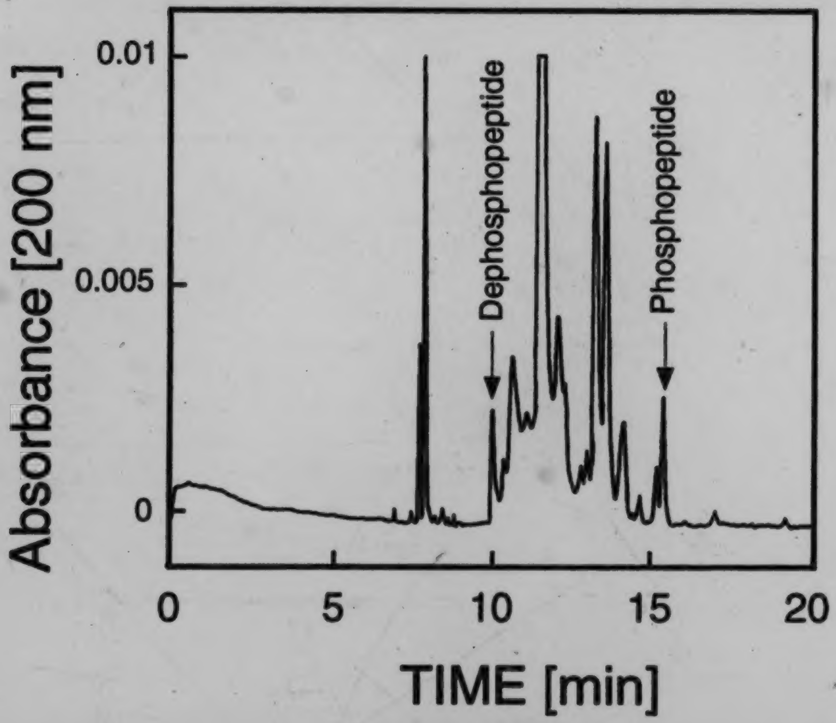


Fig. 2.3 Detection of cAMP-dependent protein kinase activity in crude rabbit skeletal muscle lysates by capillary electrophoresis. The presence of phosphorylated Kemptide in crude rabbit skeletal muscle lysates in the presence of agonists required for PKA activity was confirmed by spiking the sample with phosphorylated Kemptide standard. The total amount of Kemptide applied to the capillary was 2.6 pmol.

electrophoresis (Fig. 2.4A and 2.4B). Under these conditions, phosphorylation of PP-1G peptide on the PKA consensus sequence occurred at a faster rate.

PP-1G peptide phosphorylated simultaneously by PKA and CK-II generated single peaks of monophosphorylated and diphosphorylated products as deduced by capillary electrophoresis analysis at pH 2.0 (Fig. 2.4C). Retention times for these peptides increased concomitantly with the number of phosphorylated sites, due to the resulting increase in net negative charge of the peptide.

In contrast to pH 2.0, PP-1G peptide phosphorylated simultaneously by PKA and CK-II could be resolved by capillary electrophoresis at pH 5.0 into two species monophosphorylated on either site (in addition to unphosphorylated and diphosphorylated peptide) (Fig. 2.5). Identification of PP-1G peptide phosphorylated on Ser-9 or Ser-11 was verified by spiking the sample with PP-1G peptide phosphorylated

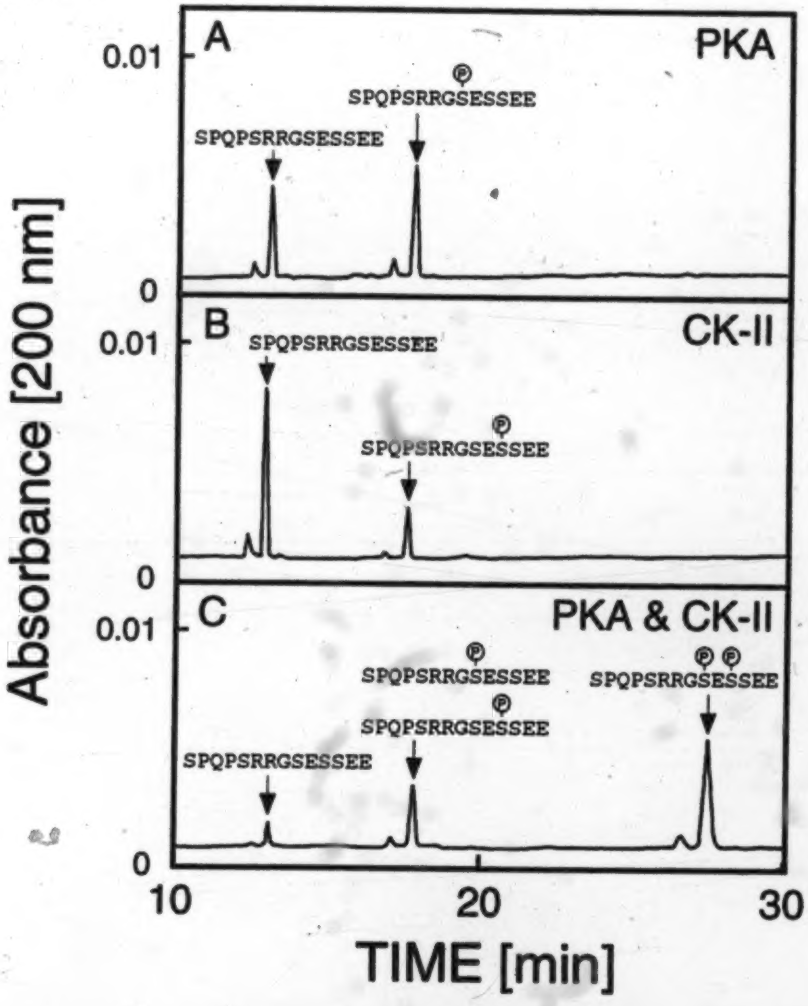


Fig. 2.4 Resolution of PP-1G peptide phosphorylated on multiple sites by cAMP-dependent protein kinase and casein kinase-II employing capillary electrophoresis at pH 2.0. All kinase reactions took place at 30° C for 10 min. A. Control phosphorylation of PP-1G by PKA (25 U) alone. B. Control phosphorylation of PP-1G by CK-II (1 mU) alone. C. Phosphorylation of PP-1G by both PKA (25 U) and CK-II (1 mU). Diphosphopeptide is resolved from monophosphopeptide species as a peak eluting at 28 min. Note that in panel C the monophosphopeptide peak is a heterogeneous mixture of PP-1G peptide phosphorylated on its PKA site and its CK-II site. The total amount of peptide applied to the capillary was 2.6 pmol.

by PKA or CK-II alone, respectively. Analysis at pH 5.0 decreased analysis time from 30 minutes to 15 minutes relative to pH 2.0 due to increased electroosmotic flow.

These results demonstrated the ability of capillary electrophoresis analysis to resolve phosphopeptides that have been phosphorylated to different degrees, i.e., phosphorylated on none, one, or two sites. Moreover, capillary electrophoresis affords the ability to monitor the phosphorylation of different sites on the same substrate simultaneously. As such, capillary electrophoresis may be employed to analyze multisite phosphorylation of peptide substrates.

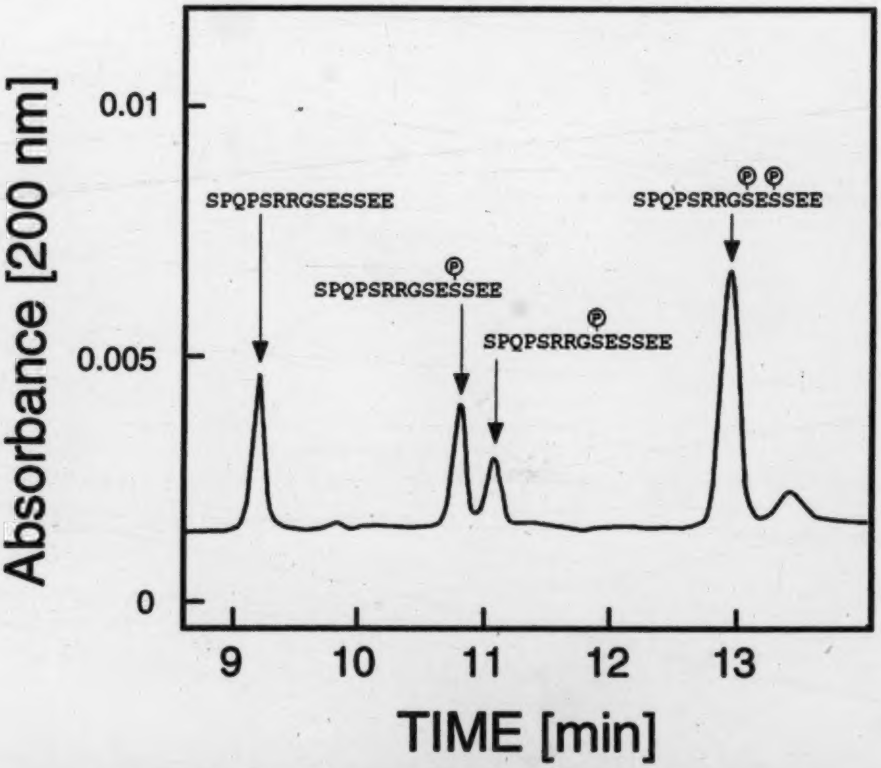


Fig. 2.5 Resolution of PP-1G peptides phosphorylated on multiple sites by cAMP-dependent protein kinase and casein kinase-II employing capillary electrophoresis at pH 5.0. The kinase reaction took place at 30 °C for 5 min in the presence of 25 U PKA and 2 mU CK-II. Unphosphorylated peptide has a retention time of 9.22 min. Diphosphopeptide phosphorylated on both PKA and CK-II sites is detected at 12.94 min. PP-1G peptide monophosphorylated by CK-II has a retention time of 10.81 min and is resolved from PP-1G peptide monophosphorylated by PKA at 11.09 min. Identification of PP-1G peptide monophosphorylated by PKA or CK-II was verified by spiking with the appropriate standard. The total amount of peptide applied to the capillary was 2.6 pmol.

Analysis of Hierarchical Phosphorylation

As discussed in section 2.1, multisite phosphorylation of proteins can occur such that phosphorylation at one site influences the ability of another site to be phosphorylated. GSK-3 has been shown to most efficiently recognize the consensus sequence Ser-Xaa-Xaa-Xaa-pSer [3] (see Table 1.1). GSK-3 is therefore capable of multiple phosphorylation that occurs in an ordered sequence, since it is able to create its own consensus sequence by phosphorylating a serine residue that is four amino acids C-terminal to another serine. This kind of ordered multisite phosphorylation is known as hierarchical phosphorylation [5] because each phosphorylation depends on the previous phosphorylation.

A fragment of glycogen synthase (termed the GS peptide, see Table 2.1) is phosphorylated *in vivo* by CK-II on Ser-21 [27]. This phosphorylation event is thought to initiate hierarchical phosphorylation by GSK-3 on Ser-17, 13, 9, and 5, in that order. Since PP-1 dephosphorylates and thereby regulates the activity of glycogen synthase, it was of interest to test the ability of the capillary electrophoresis-based assay outlined here to analyze the possible ordered multisite phosphorylation of glycogen synthase employing the GS peptide.

To test the hypothesis that glycogen synthase is phosphorylated by GSK-3 in a hierarchical fashion, GS peptide was phosphorylated by CK-II followed by GSK-3 or by GSK-3 alone and was analyzed by capillary electrophoresis at pH 5.0 to resolve different species of various degrees of peptide phosphorylation (monophosphorylated, diphosphorylated, etc.) (Fig. 2.4) . If hierarchical phosphorylation occurs, only one species of the various degrees of peptide phosphorylation should be produced. If multiple species of various degrees of phosphorylation are detected, then strict hierarchical phosphorylation is not occurring. Capillary electrophoresis analysis of kinase reactions demonstrated the presence of only single peaks of the various degrees of phosphorylation (Fig. 2.6A), thereby supporting the hypothesis that GSK-3 phosphorylates glycogen synthase in a hierarchical fashion. Phosphorylation with GSK-3 alone resulted in at least two peaks of monophosphorylated peptide and a diphosphorylated peptide possessing a different retention time than that observed with prior phosphorylation by CK-II (Fig. 2.6B).

Thus, capillary electrophoresis analysis of the phosphorylation of a fragment of glycogen synthase, a key regulator of glycogen metabolism and substrate of PP-1, provided evidence that hierarchical phosphorylation of the GS peptide by GSK-3 occurs when GS has been phosphorylated by CK-II.

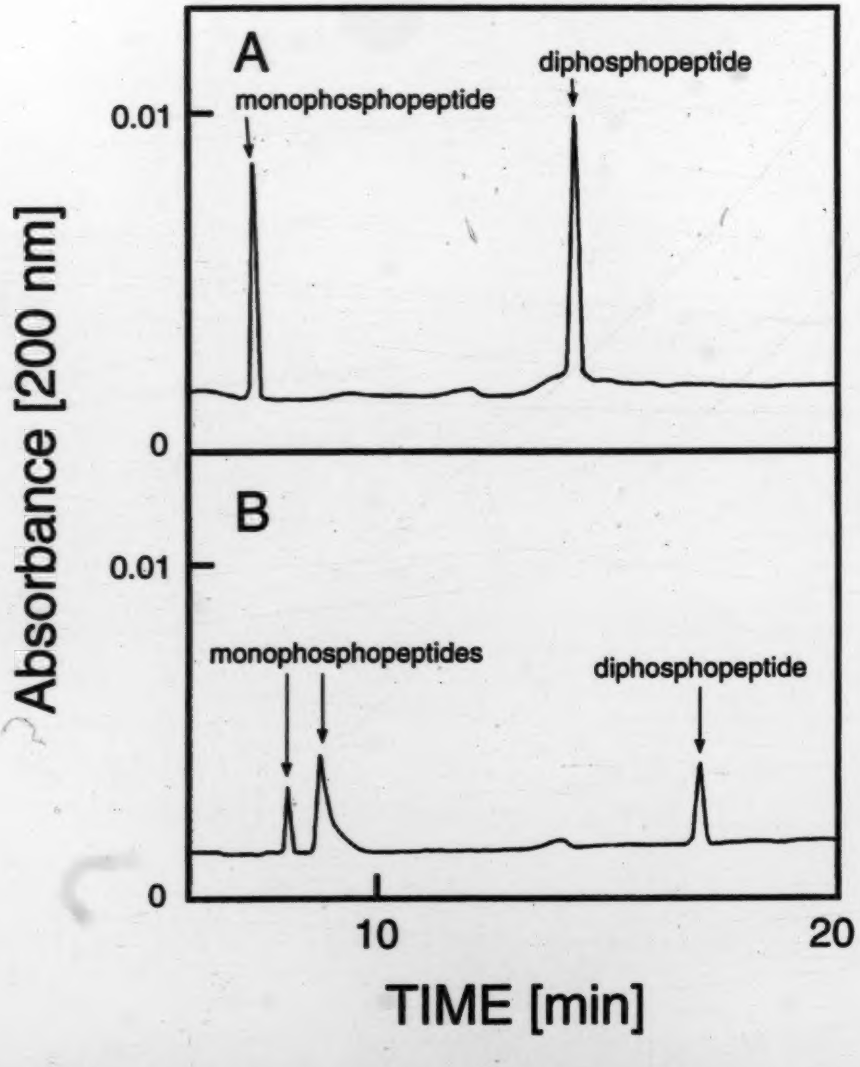


Fig. 2.6 Analysis of phosphorylation of a fragment of glycogen synthase by glycogen synthase kinase-3 and casein kinase-II using capillary electrophoresis at pH 5.0. **A.** Only single peaks of multiply phosphorylated peptide species are present when a fragment of glycogen synthase was incubated with CK-II for 2 hours prior to a 2-hour incubation with GSK-3. **B.** When phosphorylated by GSK-3 alone for 2 hours, the level of the glycogen synthase peptide fragment phosphorylation is lower. Two peaks of monophosphorylated peptide and one peak of diphosphorylated peptide are observed in this case.

Increased Detection with Laser-Induced Fluorescence

The ability of laser-induced fluorescence to increase the sensitivity of the detection of phosphorylated Kemptide standard solution was examined. At pH 2.0, phosphorylated Kemptide had a retention time of 12.6 min employing standard UV optics (Fig 2.7A). An equivalent amount of phosphorylated Kemptide was derivatized with CBQCA and analyzed in a borate buffer, pH 8.3, employing laser-induced fluorescence detection (Fig. 2.7B). LIF increased the level of sensitivity by approximately 500-fold. A very small amount of unphosphorylated Kemptide was also detected in the phosphorylated Kemptide standard solution when LIF was used. It is not possible to detect this trace amount of unphosphorylated peptide using standard UV optics.

2.4 ■ DISCUSSION

In this chapter, a capillary electrophoresis-based assay for detection of protein kinase activity based on the resolution of phosphorylated and dephosphorylated peptides was described. I chose to study phosphorylation of substrates and by kinases that are involved with PP-1 and PP-1-regulated processes. PKA, which often antagonizes the effects of PP-1, was detected in rabbit skeletal muscle extracts employing Kemptide as substrate. Furthermore, multisite phosphorylation of a fragment of the G-subunit of PP-1G, a key regulator of glycogen metabolism, was analyzed by capillary electrophoresis. The assay described above was capable of resolving PP-1G peptide phosphorylated by either PKA or CK-II. Finally, employing the capillary electrophoresis-based assay, the hypothesis of hierarchical phosphorylation of a fragment of glycogen synthase by GSK-3 was confirmed.

The use of capillary electrophoresis as a tool for the analysis of reversible phosphorylation reactions in a definable stoichiometric assay has several advantages. First, both substrate and product are resolved and simultaneously detected in reproducible and relatively short analyses. Second, this capillary electrophoresis-based assay is not dependent on modification of the peptide substrate, as is required for spectrophotometric-based [28] and fluorometric-based assays [17]. Additionally, capillary electrophoresis avoids concerns associated with spectrophotometric-based and fluorometric-based assays of background absorption or emission by compounds in a sample, especially when analyzing cell extracts. Capillary electrophoresis also circumvents the problem of background phosphorylation associated with the use of radioisotopes when using peptide substrates to detect kinase activity in cell extracts [15,16]. When radioisotopic ATP is

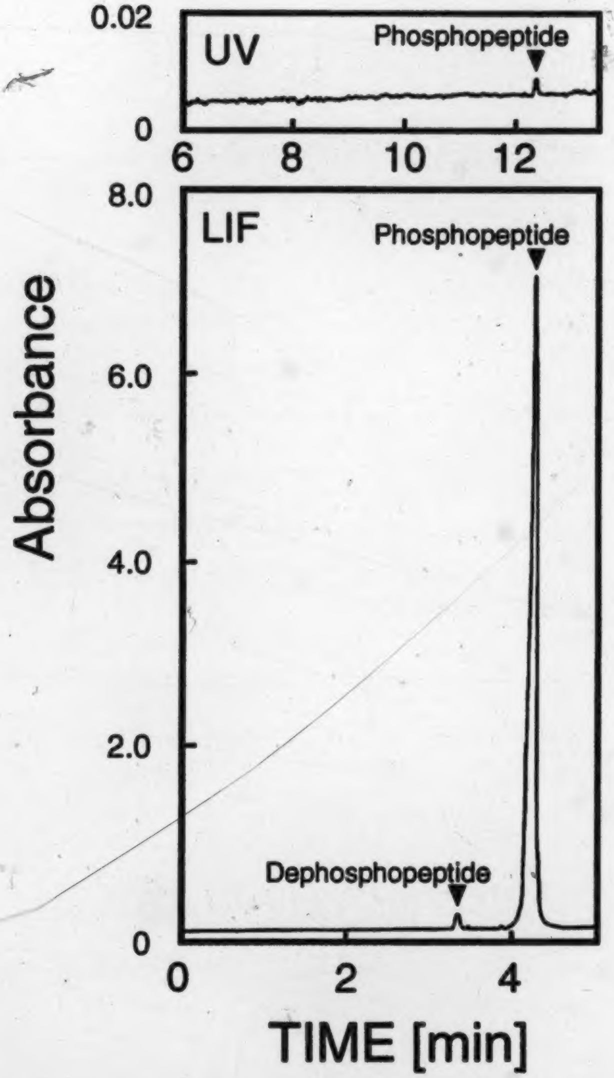


Fig.2.7 Detection of phosphorylated Kemptide by standard UV optics at 200 nm (A) versus laser-induced fluorescence (B). Equivalent amounts of Kemptide were applied to the capillary in both A and B. Standard pH 2.0 phosphoric acid buffer was employed for A, while a borate buffer pH 8.3 was using in B. LIF increases the level of detection approximately 500-fold. Note the detection of a small amount of unphosphorylated Kemptide in the phosphorylated Kemptide standard solution analyzed when LIF is used.

used, both endogenous kinase substrates and autophosphorylating kinases in addition to exogenous phosphorylated peptide substrate may be detected simultaneously. With the capillary electrophoresis-based assay, the kinase activity associated with the phosphorylation of the exogenous peptide substrate can be isolated. Without the use of radioisotopes, the capillary electrophoresis-based assay avoids concerns of isotope half-life and is safer and cheaper to perform on a per assay basis. Finally, the high resolution and reproducibility of this automated technique, combined with small sample volume requirements (usually 5 µL) and short analysis time, make capillary electrophoresis an attractive analytical method.

Analysis of the phosphorylation of peptides by capillary electrophoresis at low pH also has several advantages. Under acidic conditions, more reproducible separations occur since silica can solubilize in aqueous buffers at alkaline pH. For peptides, a pH of 2.0 is lower than their isoelectric point (pI), resulting in a net positive charge on the peptide. Under these conditions, all peptides will migrate toward the cathode and be detected. When capillary electrophoresis occurs at pH 2.0, electroosmotic flow is negligible due to the masking of negatively charged silica groups on the inner surface of the capillary by protons and separation occurs predominantly by electrophoretic forces [29,30]. Reducing electroosmotic flow results in more reproducible separations since slight variations in ionic strength and pH of the buffer and contamination of the capillary wall can occur between runs causing changes in electroosmotic flow. Since capillary electrophoresis separation occurs from anode to cathode, the presence of an additional phosphate group on a peptide confers extra negative charge at pH 2.0, resulting in longer retention times. Therefore, peptides are resolved according to their phosphorylation status.

Routinely, I found that as the pH increases, electroosmotic flow increases, resulting in decreasing retention times of the peptides. In addition, electroosmotic flow "stacks" the zone of a peptide in the capillary, resulting in sharper, taller peaks. Thus, at pH 5.0, while the resolution of phosphorylated and unphosphorylated peptide is reduced, the peaks possess greater absorbance and are sharper. The pH under which capillary electrophoresis will be carried out is therefore of utmost importance in the optimization of separation. The final decision must be a trade-off between resolution and reproducibility at low pH and short analysis time and greater absorbance of peaks at higher pH.

The resolution of PP-1G peptide phosphorylated on different sites by PKA and CK-II was not possible at pH 2.0, since both monophosphorylated peptides possess the same net overall charge. At pH 5.0, however, separation of these two peptides occurred.

Capillary electrophoresis has previously been shown to be capable of pH-dependent separation of peptides with "shuffled" amino acid sequences, possibly due to "nearest-neighbour" effects on the pK_a of charged residues [22]. While the amino acid sequence remained unchanged, the introduction of a phosphate group at different positions along the PP-1G peptide influences the charge environment surrounding the phosphoacceptor site. Therefore, nearest-neighbour effects may explain the ability of capillary electrophoresis at pH 5.0 to resolve peptides phosphorylated on different sites. Improved separation at pH 5.0 may also be explained by conformational differences between the two monophosphorylated peptides, due to different intramolecular charge interactions with the added phosphate group or increased capillary electroosmotic flow.

The capillary electrophoresis methodology described facilitates analysis of the course of simultaneous phosphorylation reactions on different sites within the same peptide substrate. This permits investigation of interdependence between different consensus sites on their respective rates of phosphorylation. I therefore examined the phosphorylation of a 27-amino-acid fragment from glycogen synthase by GSK-3 when the peptide was phosphorylated on its CK-II site or not. When prior phosphorylation by CK-II occurred, capillary electrophoresis analysis of phosphorylated forms of this peptide at pH 5.0 revealed only single peaks corresponding to multiply phosphorylated forms of this peptide, clearly supporting the hypothesis that phosphorylation of glycogen synthase by GSK-3 follows a sequential pattern. Without prior phosphorylation by CK-II, only small amounts of phosphopeptide were detected, confirming the observation that unphosphorylated glycogen synthase is a poor GSK-3 substrate [3]. In addition, multiple peaks of monophosphopeptide were detected, demonstrating that in the absence of CK-II site phosphorylation, phosphorylation of glycogen synthase by GSK-3 does not follow a sequential pattern. The ability of capillary electrophoresis to analyze hierarchical phosphorylation of glycogen synthase by GSK-3 suggests that this method may prove to be extremely useful for the analysis of peptides that are phosphorylated at multiple sites *in vivo*.

The sensitivity of the capillary electrophoresis-based protein kinase assay in this study was approximately 10-fold less than that of radioisotopic assays [15,16] when UV optics were employed. However, the use of a laser-induced fluorescence (LIF) detection system enhances sensitivity approximately 500-fold that of standard UV optics. LIF-capillary electrophoresis may ultimately provide greater sensitivity of detection over radioisotopic assays, with possible detection limits in the attomolar range [24]. However, given the prohibitive cost of a laser-induced detection system, LIF-capillary electrophoresis may not be exploited to its full potential.

2.5 ■ BIBLIOGRAPHY

1. Hunter, T. (1995) Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signalling. *Cell* **80**: 225-236.

2. DePaoli-Roach, A. A., Ahmad, Z., and Roach, P. J. (1981) Characterization of a rabbit skeletal muscle protein kinase ($PC_{0.7}$) able to phosphorylate glycogen synthase and phosvitin. *J. Biol. Chem.* **256**: 8955-8962.

3. Fiol, C. J., Wang, A., Roeske, R. W., and Roach, P. J. (1990) Ordered multisite protein phosphorylation. *J.Biol. Chem.* **265**: 6061-6065.

4. Wang, Y., and Roach, P. J. (1993) Inactivation of rabbit muscle glycogen synthase by glycogen synthase kinase-3. *J. Biol. Chem.* **268**: 23876-23880.

5. Roach, P. J. (1991) Multisite and hierarchical protein phosphorylation. *J. Biol. Chem.* **266**: 14139-14142.

6. Kennelly, P. J., and Krebs, E.G. (1991) Consensus sequences as substrate specific determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**: 15555-15558.

7. Pearson, R. B., and Kemp, B. E. (1991) Protein kinases phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol.* **200**: 62-81.

8. Kemp, B. E., and Pearson, R. B. (1991) Design and use of peptide substrates for protein kinases. *Methods Enzymol.* **200**: 121-134.

9. Kemp, B. E., Benjamini, E., and Krebs, E. G. (1976) Synthetic hexapeptide substrates and inhibitors of 3':5'-cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **73**: 1038-1042.

10. Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **252**: 4888-4894.

11. Maller, J. L., Kemp, B. E., and Krebs, E. G. (1978) *In vivo* phosphorylation of a synthetic peptide substrate of cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **75**: 248-251.

12. Zetterqvist, Ö., Ragnarsson, U., and Engström, L. (1990) in *Peptides and protein phosphorylation* (Kemp, B. E., ed), pp. 171-188, CRC Press, Boca Raton

13. Caudwell, F. B., Hiraga, A., and Cohen, P. (1986) Amino acid sequence of a region on the glycogen-binding subunit of protein phosphatase-1 phosphorylated by cyclic AMP-dependent protein kinase. *FEBS Lett.* **194**: 85-90.

14. Marin, O., Meggio, F., Marchiori, F., Borin, G., and Pinna, L. A. (1986) Site specificity of casein kinase-2 (TS) from rat liver cytosol. *Eur. J. Biochem.* **160**: 239-244.

15. Glass, D. B., Masaracchia, R. A., Feramisco, J. R., and Kemp, B. E. (1978) Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal. Biochem.* **87**: 566-575.

16. Casnellie, J. E. (1991) Assay of protein kinases using peptides with basic residues for phosphocellulose binding. *Methods Enzymol.* **200**: 115-120.

17. Wright, D. E., Noiman, E. S., Chock, P. B., and Chau, V. (1981) Fluorometric assay for adenosine 3',5'-cyclic monophosphate-dependent protein kinase and phosphoprotein phosphatase activities. *Proc. Natl. Acad. Sci. USA* **78**: 6048-6050.

18. Tiselius, A. (1937) A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans. Faraday Soc.* **33**: 524-531.

19. Hjerten, S. (1967) Free zone electrophoresis. *Chromatogr. Rev.* **9**: 122-219.

20. Kuhr, W. G. (1990) Capillary electrophoresis. *Anal. Chem.* **62**: 403R-414R.

21. Landers, J. P., Oda, R. P., Spelsberg, T. C., Nolan, J. A., and Ulfelder, K. J. (1993) Capillary electrophoresis: a powerful microanalytical technique for biologically active molecules. *Biotechniques* **14**: 98-111.

22. Landers, J. P. (1993) Capillary electrophoresis: pioneering new approaches for biomolecular analysis. *Trends Biochem. Sci.* **18**: 409-414.

23. Rabel, S. R., and Stobaugh, J. F. (1993) Pharmaceutical applications of capillary electrophoresis. *Pharm. Res.* **10**: 171-186.

24. Cobb, K. A., and Novotny, M. V. (1992) Selective determination of arginine-containing and tyrosine-containing peptides using capillary electrophoresis and laser-induced fluorescence detection. *Anal. Biochem.* **200**: 149-155.

25. Skeidsvoll, J., and Ueland, P. M. (1995) Analysis of double-stranded DNA by capillary electrophoresis with laser-induced fluorescence detection using the monomeric dye SYBR green I. *Anal. Biochem.* **231**: 359-365.

26. Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Straalfors, P., and Tung, H. Y. L. (1988) Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Methods Enzymol.* **159**

27. Fiol, C. J., Mahrenholz, A. M., Wang, Y., Roeske, R. W., and Roach, P. J. (1987) Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase-II and glycogen synthase kinase-3. *J. Biol. Chem.* **262**: 14042-14048.

28. Roskiski, R. (1983) Assays of protein kinases. *Methods Enzymol.* **99**: 3-6.

29. McCormick, R. M. (1988) Capillary zone electrophoresis separation of peptides and proteins using low pH buffers in modified silica capillaries. *Anal. Chem.* **60**: 2322-2328.

30. Wu, C.-T., Lopes, T., Patel, B., and Lee, C. S. (1992) Effect of direct control of electroosmosis on peptide and protein separations in capillary electrophoresis. *Anal. Chem.* **64**: 886-891.

Chapter 3

Signal Transduction by Protein Kinases and Phosphatases in Marine Dinoflagellates:

Part I:
Protein Tyrosine Kinases[§] and Phosphatases[*]

Part II:
Protein Serine/Threonine Kinases[§]

Parts of this chapter have already been published:

[*] Dawson, J. F., Ostergaard, H. L., Klix, H., Boland, M. P., and Holmes, C. F. B. (1997) Evidence of Reversible Tyrosine Protein Phosphorylation in the Okadaic Acid-Producing Marine Dinoflagellate *Prorocentrum lima*. J. Euk. Microbiol. **44**: 89-95.

[§] Dawson, J. F., Wang, H. E., and Holmes, C. F. B. (1996) Identification and characterization of cAMP-dependent protein kinase and its possible direct interactions with protein phosphatase-1 in marine dinoflagellates. Biochem. Cell Biol. **74**: 559-567.

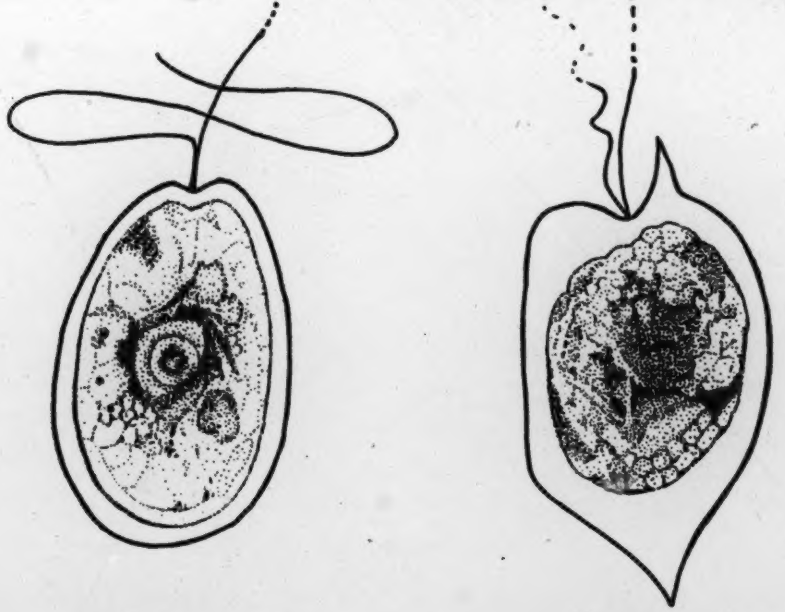
3.1 ■ INTRODUCTION

Reversible protein phosphorylation is an essential part of the process of signal transduction which modulates how cells respond to external stimuli. The level of protein phosphorylation in the cell is tightly regulated by the concerted and antagonistic actions of protein kinases and protein phosphatases [1,2]. Protein kinases catalyze the transfer of the γ phosphate from ATP to the hydroxyl groups of serine, threonine, or tyrosine side chains, thereby effecting a change in the structure of target proteins [3]. Conversely, protein phosphatases catalyze the removal of phosphate groups from phosphoserine, phosphothreonine, or phosphotyrosine and are therefore essential to the reversible nature of protein phosphorylation [4-6]. As such, understanding how protein phosphatase activity is regulated is of vital importance to our appreciation of reversible protein phosphorylation, signal transduction and, more generally, how cells adapt to changes in their environment.

A major objective of my work is to provide an understanding of how protein phosphatase-1 (PP-1) is regulated by a growing family of natural product toxins. These include potent tumour promoters produced by marine and freshwater algae. We hypothesized that we might gain insight into how algal toxins regulate PP-1, and moreover signal transduction involving reversible protein phosphorylation, by studying signal transduction in organisms that are primary producers of these toxins. One such organism is *Prorocentrum lima*, a marine dinoflagellate.

Marine Dinoflagellates

Marine dinoflagellates, classified as Protists of the Division Pyrrophyta, are a diverse assemblage of biflagellar unicellular organisms inhabiting both marine and freshwater environments. Blooms of marine dinoflagellates, sometimes manifested in the phenomenon known as "red tide", are associated with the production of toxins. Of particular interest are the marine Prorocentrale species (Fig. 3.1), particularly *Prorocentrum lima*, which is a primary producer of okadaic acid [7,8], a potent serine/threonine protein phosphatase inhibitor [9] and tumour promoter [10,11] that causes diarrhetic shellfish poisoning in humans [12]. This has raised the question of how okadaic acid-producing marine dinoflagellates such as *P. lima* protect themselves from such a potent phosphatase inhibitor and what is the nature of signal transduction involving protein kinases and phosphatases in these organisms.



Prorocentrum lima *Prorocentrum micans*

Fig. 3.1 *Prorocentrum lima* and *Prorocentrum micans*. The order Prorocentrales is characterized by a thick armoured cell wall composed mainly of cellulose and known as a theca, which consists of two plates, or valves. The name *Prorocentrum* (latin *prora*, prow, and latin *centrum*, center) is derived from the general teardrop shape of many of these dinoflagellates in face view, shown above. Laterally, Prorocentrale dinoflagellates exhibit a compressed form. An anterior spine is present on some (e.g. *Prorocentrum micans*), but not all (e.g. *Prorocentrum lima*) members of this order. Unlike most dinoflagellates, possessing two flagella situated in grooves on the thecal plates, one directed posteriorly and another encircling the cell equatorially, the two flagella of Prorocentrale dinoflagellates both emerge anteriorly. One flagellum is directed along the long axis of the cell while its partner encircles the other and is oriented perpendicularly. Reproduction of Prorocentrale dinoflagellates occurs by longitudinal division of a cell into two cells, each keeping one of the parental plates. *Prorocentrum lima* is of particular interest because it is a primary producer of okadaic acid, a potent inhibitor of protein phosphatases-1 and 2A, as discussed in section 1.6. Conversely, two other dinoflagellates examined in the work described here, *Prorocentrum micans* and *Prorocentrum minimum* (not shown), do not produce okadaic acid. This has raised the question of how okadaic acid-producing marine dinoflagellates protect themselves from the toxin they produce, as discussed in the text. The illustrations depicted here are of dinoflagellates cultured in the laboratory at the University of Alberta. Magnification is approximately 500x.

It has been hypothesized that marine dinoflagellates are mesokaryotic, i.e., intermediate between prokaryotes and eukaryotes, based on early observations that dinoflagellate DNA lacked histones and nucleosomes, mitosis occurred via a membranous mechanism, and DNA was replicated continuously [13]. Fossil records suggest that marine dinoflagellates are a very ancient group which may have diverged from the eukaryotic organization more than 900 million years ago when the first marine dinoflagellates existed or perhaps 2 billion years ago when the first possible eukaryotes occurred [14].

Marine Dinoflagellate Toxin Resistance

To account for the apparent resistance of marine dinoflagellates to the toxins they produce, it has been hypothesized that the phosphatases in these organisms may be of a more primitive or altogether different origin than those of their mammalian counterparts, in accordance with the mesokaryotic hypothesis. Yet another theory holds that toxin-producing cells may compartmentalize the toxins away from the phosphatases.

Recently, more detailed evidence revealed that mitosis in marine dinoflagellates involves microtubules and that they possess a definite cell cycle including a DNA replication phase [15]. In addition, accumulating ribosomal RNA sequencing data suggests that the marine dinoflagellates emerged late in eukaryotic evolution in a cluster with the ciliates (Ciliophora) and sporozoans (Apicomplexa) [16]. In addition, it was determined during the time of my work that a number of Prorocentrale species possess protein phosphatases-1 and 2A [17,18], both biochemically similar to their mammalian homologs. As such, evidence is accumulating to suggest that marine dinoflagellates are not as primitive as once thought and that they are in fact true eukaryotes that have secondarily lost a gene cluster that encodes histone proteins, accounting for their unique chromosomal and nuclear structure.

Immunolocalization experiments have recently demonstrated that okadaic acid is localized in peripheral chloroplasts and autodigestive lysosomes (PAS bodies) and may be associated with membrane lipids [19]. Therefore, the hypothesis of toxin sequestration appears to be the most viable presently, although a potential caveat to this study is that the antibody employed may not recognize okadaic acid bound to PP-1.

The objective of the work described in this chapter was to investigate the nature of signal transduction by protein kinases and phosphatases in marine dinoflagellates and specifically the okadaic acid-producing species *P. lima* and thereby provide an understanding of how okadaic acid might play a role in regulating reversible protein phosphorylation in this organism and, secondarily, provide insight into the phylogenetic position of marine dinoflagellates. The work described extends our body of knowledge regarding marine dinoflagellate biochemistry with regard to protein kinase and phosphatase signaling that occurs in these marine organisms and lends support to the placement of marine dinoflagellates within the eukaryotic organization.

3.2 ■ PART I: PROTEIN TYROSINE KINASES AND PHOSPHATASES IN MARINE DINOFLAGELLATES

Reversible protein phosphorylation on tyrosine residues, regulated by the concerted actions of protein tyrosine kinases (PTKs) [20,21] and protein tyrosine phosphatases (PTPases) [22,23], is of primary importance to growth and development of eukaryotic cells and is characteristic of phosphotyrosine signaling in most cases.

Initially, lack of evidence for reversible tyrosine protein phosphorylation among unicellular eukaryotes suggested that reversible tyrosine protein phosphorylation evolved among metazoans as a mechanism to coordinate multicellular growth [3]. Today, components of phosphotyrosine signaling have been identified in unicellular organisms such as fission yeast [24-28] and trypanosomes [29,30] as well as higher plants [31-34].

In part one of this chapter, I present evidence for reversible protein tyrosine phosphorylation in marine dinoflagellates and specifically, the okadaic acid-producing *Prorocentrum lima*. In an effort to uncover signaling pathways in which okadaic acid may play a physiological role, I searched for protein kinase activity in this organism. Herein is described the identification and characterization of an autophosphorylating protein tyrosine kinase in *P. lima* cell extracts referred to as PLIK 1A (*P. lima* kinase 1A). The presence of protein tyrosine kinase activity in *P. lima* lends support to the hypothesis that marine dinoflagellates are eukaryotes, since regulated phosphorylation of tyrosine residues is not known to occur in prokaryotes. The identification of a cdc2-like kinase, a key regulator of eukaryotic cell cycle control (discussed in section 1.3), in the dinoflagellate *Crypthecodinium cohnii* [35] and the observation that growth rates of certain dinoflagellates increase upon exposure to platelet derived growth factor and phorbol esters [36] strengthens the hypothesis that growth control mechanisms in dinoflagellates may be analogous to those in higher eukaryotes. Herein, I present immunological evidence that *P. lima* also possesses a cdc2-like kinase, thereby including the Prorocentrale marine dinoflagellates in the hypothesis described above.

The presence of protein tyrosine kinase activity in *P. lima* also suggested the possibility of PTPase activity in Prorocentrale species. I therefore searched for PTPase activity and outline the identification and characterization of two PTPase activities in *P. lima*, referred to herein as PLPTP-I and PLPTP-II (*P. lima* PTPase). Additionally, PTPase activity was identified and characterized in two other non-okadaic acid-producing Prorocentrale species, *P. micans*, and *P. minimum*. Finally, it is demonstrated that both

PLPTP-I and II are able to employ autophosphorylated PLIK 1A as a phosphotyrosine-containing substrate *in vitro.*

3.3 ■ MATERIALS AND METHODS

Cell Culture and Strain

Prorocentrale cells were cultured as described in [17]. Benthic marine dinoflagellate isolates *P. lima* strain 712, *P. micans* strain 433, and *P. minimum* strain 514 were from the North Eastern Pacific Culture Collection (NEPCC), Department of Oceanography, University of British Columbia.

Preparation of Extracts for Protein Kinase Assays

Cells were harvested by centrifugation at 1250 *g* (r_{av}=18.4 cm). Extraction buffer (3 mL) comprising 50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol, 20% glycerol (v/v), 0.5 mM PMSF, 0.5 mg/mL leupeptin, and 1.4 mg/mL pepstatin was added per gram of wet weight cell pellet. Cell suspensions were sonicated with four pulses (30 sec) with equal rest periods on ice. Resulting homogenates were centrifuged at 16000 *g* (r_{av}=7.3 cm) for 20 min at 4 °C. Supernatants were then flash frozen and stored at -80 °C.

Protein Kinase Assays

Marine dinoflagellate extracts were fractionated utilizing a Mono-Q anion exchange column (Pharmacia, Uppsala, Sweden) developed with a linear gradient from 0 to 500 mM NaCl in running buffer (20 mM triethanolamine-HCl, pH 7.0, 0.1 mM EGTA, and 0.1 mM DTT). Aliquots (50 µL) of Mono-Q fractions (1 mL) were preincubated at 30 °C for 5 min. Kinase reactions were initiated with the addition of 5 µL of a kinase reaction mixture containing 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, and 0.1 mCi [γ-^{32}P] ATP (Amersham, Oakville, Canada) at 30 °C. Reactions were quenched after 15 min by spotting 50 µL of the reaction onto P-81 phosphocellulose paper (Whatman), or by the addition of SDS-PAGE sample buffer (30 µL) followed by 5 min boiling prior to SDS-PAGE (12% acrylamide). Phosphocellulose filters were washed twice in 0.75 M H_3PO_4 for 5 min and counted using a scintillation counter (1209 rackbeta, Wallac). Gels were exposed to X-ray film (Kodak, Rochester, NY) and an autoradiogram was produced.

Antiphosphotyrosine Western Immunoblotting

Mono-Q fractions (100 µL) developed as described above were incubated in a kinase reaction mixture (10 µL) comprising 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 1 mM ATP at 30 °C for 15 min. Reactions were quenched by the addition of SDS-PAGE sample buffer (40 µL) followed by boiling for 5 min prior to SDS-PAGE. Protein was then transferred to nitrocellulose and probed with an antiphosphotyrosine monoclonal antibody (UBI, Lake Placid, NY).

Preparation of Cell Extracts for Tyrosine Phosphatase Assays

Phosphatase extraction buffer (4 mL) comprising 50 mM Tris-HCl, pH 7.2, 5 mM EDTA, 10 mM DTT, 0.05% Brij 35 (v/v), and a cocktail of protease inhibitors (described in [17], except that PefaBloc replaced APMSF) was added per gram wet weight cell pellet. Cell suspensions were then passed through a French press twice at 16000 psi. Resulting homogenates were then centrifuged at 16000 *g* (r_{av}=7.3 cm) for 20 min at 4 °C.

p-Nitrophenolphosphate Phosphatase Assays

Marine dinoflagellate extracts were fractionated by a Mono-Q protocol that was similar to that used for autophosphorylating protein kinase analysis except that the column was developed with a gradient from 0 to 1M NaCl. Protein phosphatase assays were performed using *p*NPP (*p*-nitrophenolphosphate) essentially as described in [37] with the following modifications: the sample to be assayed was added to assay buffer consisting of 25 mM imidazole, pH 7.2, 1 mM EDTA, and 0.1 mM DTT and either 200 µM Na_2MoO_4 or 1 mM $ZnCl_2$ as inhibitors to a total volume of 90 µL in a microtiter plate well. *p*NPP (10 µL of 1.5 mg/mL) was added to start the reaction and the plate was incubated at 37 °C for 60 min. Plates were then scanned at 405 nm employing a microplate reader.

Protein Tyrosine Phosphatase Assays

Isotopic protein tyrosine phosphatase assays were performed essentially as described in [37]. Briefly, myelin basic protein (MBP) or angiotensin was incubated overnight with $p56^{lck}$ in the presence of 100 µCi [γ-^{32}P] ATP (ICN, Montreal, Canada). Tyrosine-phosphorylated protein was recovered after trichloroacetic acid precipitation in the presence of bovine serum albumin carrier protein. Tyrosine-phosphorylated MBP or angiotensin was mixed with PLPTP-I or II in 25 µM imidazole, pH 7.2, 5 mM EDTA, and 100 mM DTT . Reactions were stopped at the appropriate time with acidic charcoal, which was pelleted by centrifugation after 10 min at room temperature. Released ^{32}P was quantitated in the supernatants by Cerenkov counting.

Superdex Gel Filtration Chromatography

Active PLIK 1A Mono-Q fractions were rechromatographed on a Superdex-75 gel filtration column with 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 0.1 mM EGTA, and 0.1 mM DTT . Active pools of PLPTP-I and II were developed on a Superdex-200 gel filtration column under the same conditions. Molecular mass standards (13.7 to 669 kDa) were from Pharmacia (Uppsala, Sweden).

PLIK 1A Dephosphorylation Assays

Active PLIK 1A Mono-Q fractions were incubated in a kinase reaction mixture of 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 2 µCi [γ-^{32}P] ATP for 60 min at 30 °C followed by a 10 min incubation at 4 °C. Active PLPTP-I or II fractions (30 µL) were added to autophosphorylated PLIK 1A (20 µL), incubated for 1 h, and analyzed employing 12% SDS-PAGE.

Anti-cdc2 kinase Western Immunoblotting

To an aliquot (200 µL) of a *P. lima* cell extract produced employing a French press as described above was added 1 mL of Tris-buffered phenol, pH 7.6. Following centrifugation in a benchtop centrifuge at room temperature for 5 min, the phenol phase was removed and added to 5 mL of a 4:1:1 mixture of acetone, ethanol, and water. The mixture was then incubated at -20 °C overnight and centrifuged at 16000 *g* (r_{av}=7.3 cm) for 5 min at 4 °C. The resulting pellet was resuspended in 4.5 mL of the 4:1:1 mixture of acetone, ethanol, and water and collected by centrifugation. The pellet was then washed four times in ice-cold ethanol prior to the addition of SDS-PAGE sample buffer and incubation at 85 °C for 10 min. Protein extracts prepared in this manner were run on 12% SDS-PAGE alongside 25 µL of an *S. pombe* cell extract (a gift from C. Rasmussen, University of Alberta). Protein was then transferred to nitrocellulose and probed with an anti-cdc2 kinase monoclonal antibody directed against the PSTAIR motif (Boehringer-Mannheim).

3.4 ■ RESULTS

Identification and Characterization of an Autophosphorylating Protein Tyrosine Kinase in *Prorocentrum lima*.

While analyzing whole cell extracts from *Prorocentrum lima* by Mono-Q anion exchange chromatography for the presence of protein kinase activity, several column fractions were identified that became radiolabelled when incubated with [γ-^{32}P] ATP (Fig. 3.2A). Radiolabelled fractions from Mono-Q chromatography were analyzed by SDS-PAGE and autoradiographed and correspond to a protein with an apparent molecular mass of 38 kDa (Fig. 3.2B), referred to herein as PLIK 1A (*P. lima* kinase 1A). Other radioactive bands were also observed following SDS-PAGE and autoradiography in later Mono-Q fractions, most notably at apparent molecular masses of approximately 39 kDa, 46 kDa and 54 kDa. The nature of these proteins is presently unknown, but may represent other autophosphorylating protein kinases. To exclude the possibility that an ATP-binding protein was responsible for PLIK 1A activity, the fractions from Mono-Q chromatography that contained PLIK 1A activity were incubated with [α-^{32}P] ATP. These fractions demonstrated no significant retention of radioactive signal.

To determine which residues on PLIK 1A were autophosphorylated, active fractions were incubated with [γ-^{32}P] ATP and subjected to phosphoamino acid analysis by acid hydrolysis of the protein followed by thin layer chromatography analysis [38]. These results indicated that PLIK 1A autophosphorylates almost exclusively on tyrosine residues. To confirm this initial observation, active PLIK 1A Mono-Q fractions were incubated with ATP and magnesium to produce Western immunoblots (Fig. 3.2C). The protein band corresponding to PLIK 1A in SDS-PAGE autoradiograms at 38 kDa also reacted with the monoclonal anti-phosphotyrosine antibodies in the Western blot analysis. These results strongly suggest that PLIK 1A represents an autophosphorylating protein tyrosine kinase.

Partially purified PLIK 1A from Mono-Q chromatography was fractionated further on a Superdex-75 gel filtration column. The resulting column fractions were then assayed for the presence of autophosphorylating protein kinase activity employing the phosphocellulose-based assay (Fig. 3.3). Two peaks of activity were observed, eluting within a molecular mass range of 47 to 55 kDa and the second in the range of 35 to 40 kDa. The second peak of activity corresponded well with the observed molecular mass from autoradiograms following SDS-PAGE analysis. These results suggest that PLIK

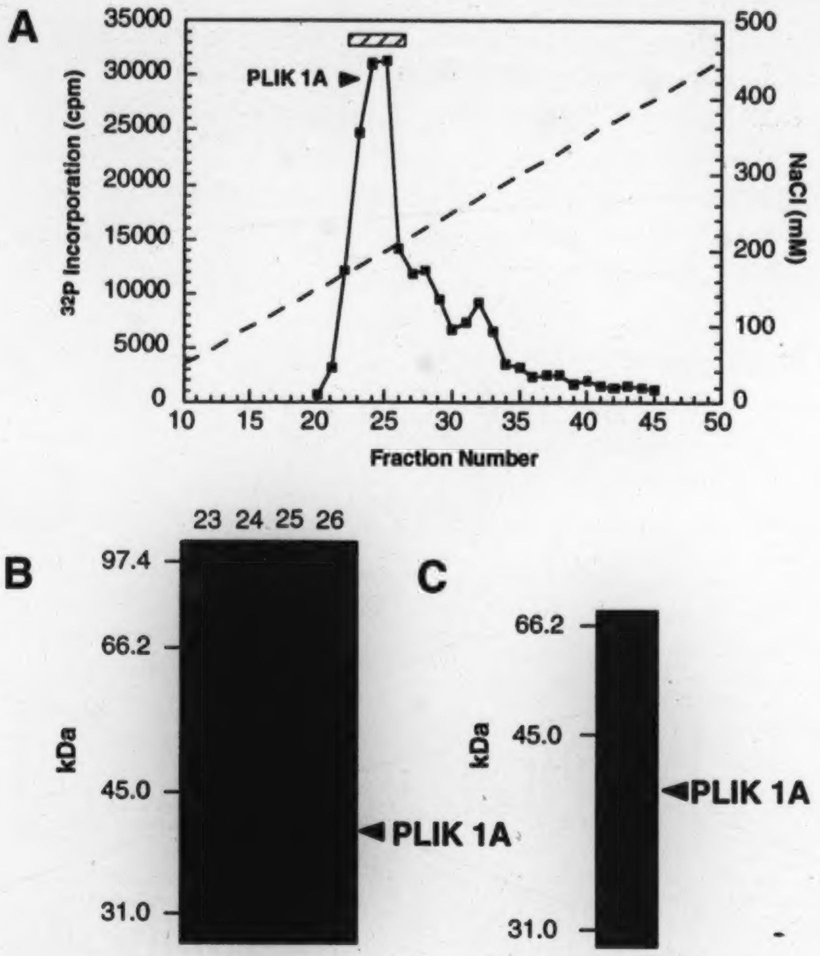


Fig. 3.2 Identification of an autophosphorylating activity in Mono-Q fractions of a *Prorocentrum lima* cell extract. A. Aliquots (50 µL) of Mono-Q fractions (1 mL) were incubated with [γ-^{32}P] ATP and filtered through phosphocellulose, washed, and counted in a scintillation counter. Fractions indicated by the hatched bar were incubated with [γ-^{32}P] ATP, analyzed by SDS-PAGE (12%) and an autoradiogram produced (B). Peaks of activity detected in fractions 23 to 25 were termed PLIK 1A. The autophosphorylating activity in these fractions is detected as a band on the autoradiogram with an apparent molecular mass of approximately 38 kDa. C. Western immunoblot of PLIK 1A incubated with cold ATP, analyzed by SDS-PAGE (12%), transferred to nitrocellulose and immunoblotted with a monoclonal antiphosphotyrosine antibody (UBI, Lake Placid, NY). Identification of a band corresponding to PLIK 1A confirms that it is phosphorylated on tyrosine residues.

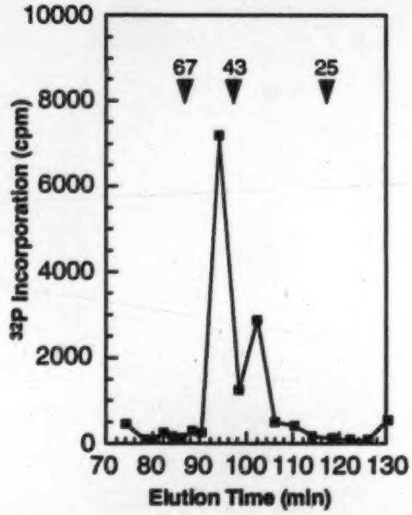


Fig. 3.3 Resolution of PLIK 1A into two components by Superdex-75 gel filtration chromatography. Aliquots (50 µL) of Superdex-75 gel filtration fractions (200 µL) of an active PLIK 1A Mono-Q fraction were incubated with [γ-^{32}P] ATP, filtered through phosphocellulose, washed and counted in a scintillation counter. Two peaks of activity eluted within the native molecular mass ranges of 47 to 55 kDa and 35 to 40 kDa based on the elution position of relevant molecular mass standards shown across the top in kDa. The second peak is in agreement with the SDS-PAGE apparent molecular mass of approximately 38 kDa.

1A may be present in two forms following Mono-Q chromatography both of which contain an autophosphorylating protein tyrosine kinase subunit of approximately 38 kDa.

Identification and Characterization of Protein Tyrosine Phosphatases in Prorocentrale Species

Mono-Q fractions from *P. lima* cell extracts were assayed for their ability to dephosphorylate the general phosphatase substrate *p*-nitrophenolphosphate (*p*NPP) (Fig. 3.4A). These experiments revealed the presence of two distinct phosphatase activities, termed PLPTP-I and PLPTP-II, that could be inhibited by the classical protein tyrosine phosphatase inhibitors molybdate (200 µM) [39] and zinc (1 mM) [40], although PLPTP-II was less sensitive to inhibition by either agent, particularly molybdate. Conversely, neither tetramisole (1 mM) (an inhibitor of alkaline phosphatase activity [41]), tartrate (an inhibitor of some acid phosphatases [42]), nor microcystin LR (5 µM) (a potent inhibitor of type-1 and -2A protein serine/threonine phosphatases [43]) produced inhibition of PLPTP-I or II (Table 3.1). Sodium fluoride (200 µM) had no reproducible effect on PLPTP-I and II activity. Both PLPTP-I and II were analyzed by Superdex-200 gel filtration chromatography (Fig. 3.4B and C). The PLPTP-I peak consisted of a single

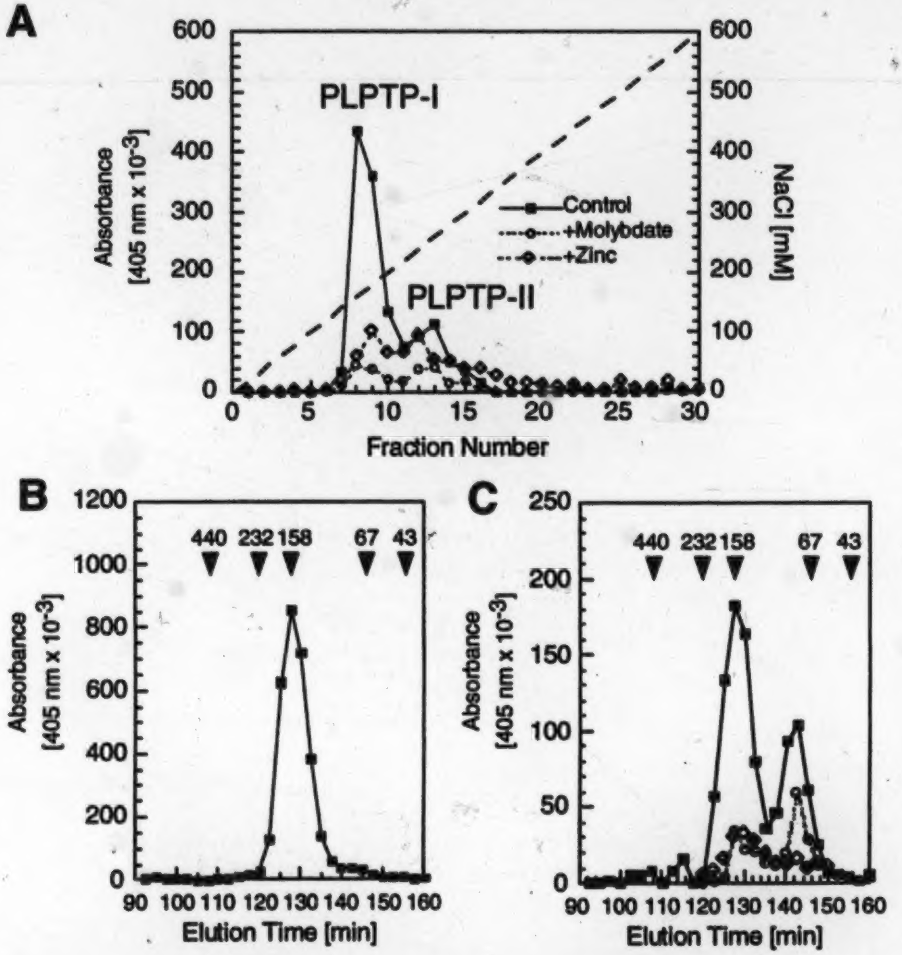


Fig. 3.4 Identification and characterization of protein tyrosine phosphatase activities in *Prorocentrum lima* cell extracts. A. Identification of protein tyrosine phosphatase activities in Mono-Q fractions of a *P. lima* cell extract. Aliquots (20 µL) of each fraction (1 mL) were incubated for 1 hr at 37 °C in the presence or absence of either 200 µM molybdate or 1 mM zinc employing 15 µg *p*NPP as substrate. The release of phosphate from *p*NPP was measured as a function of the absorbance of the product *p*-nitrophenol at 405 nm. Two peaks of activity were observed, termed PLPTP-I and PLPTP-II according to their elution position. B. Superdex-200 gel filtration analysis of the PLPTP-I peak. Superdex-200 gel filtration fractions (200 µL) were assayed as above. PLPTP-I eluted within the molecular mass ranges of 150 to 168 kDa based on the elution position of relevant molecular mass standards shown across the top in kDa. C. Superdex-200 gel filtration analysis of the PLPTP-II peak. Superdex-200 fractions were analyzed employing molybdate and zinc as inhibitors as above. Data points are denoted as in A. The two peaks, corresponding to molecular mass ranges of 150 to 168 kDa and 73 to 82 kDa based on the elution position of relevant molecular mass standards shown in kDa, demonstrate differential inhibition by molybdate.

activity eluting from the gel filtration column within the molecular mass range of 150 to 168 kDa, while PLPTP-II possessed two peaks of activity eluting within molecular mass ranges of 150 to 168 kDa and 73 to 82 kDa. The lower molecular mass activity displayed reduced sensitivity to inhibition by molybdate in particular while the enzymatic activity corresponding to the larger molecular mass peak exhibited inhibition similar to PLPTP-I.

Since *P. lima* is a primary producer of okadaic acid, it was important to determine whether two other Prorocentrale marine dinoflagellates that do not produce okadaic acid, *P. micans* and *P. minimum*, also possess PTPase activity. Cell extracts from each species were chromatographed by Mono-Q anion exchange chromatography and fractions assayed for PTPase activity. Two major peaks of phosphatase activity were identified in *P. micans*, termed PMPTP-I and PMPTP-II (Fig. 3.5A). These activities exhibited significant resistance to the panel of inhibitors tested (Table 3.1), although there was limited sensitivity to molybdate or zinc in each case, indicative of protein tyrosine phosphatases. PMPTP-I and PMPTP-II responded differently to molybdate and zinc, suggesting that they may represent two distinct PTPase activities in this marine dinoflagellate. Similarly, two peaks of phosphatase activity were identified in *P. minimum* referred to as PNPTP-I and PNPTP-II (Fig. 3.5B). Both activities responded in a similar fashion to the panel of inhibitors employed to characterize the PLPTPases described above (Table 3.1), demonstrating inhibition in the presence of molybdate and zinc, characteristic of protein tyrosine phosphatases.

Table 3.1 Sensitivity of Prorocentrale protein tyrosine phosphatases activities to inhibition by various phosphatase inhibitors. Numbers shown are the percentage of the activity of a phosphatase relative to control activity with no inhibitor added. Each number represents the average of triplicate trials.

	P. lima		*P. micans*		*P. minimum*	
	PLPTP-I	PLPTP-II	PMPTP-I	PMPTP-II	PNPTP-I	PNPTP-II
Molybdate 0.2 mM	4	34	26	29	69	89
Zinc 1 mM	26	112	68	67	103	72
NaF 30 mM	102	77	41	60	91	117
Microcystin-LR 1 µM	97	90	103	92	115	94
Tartrate 3 mM	102	86	89	77	104	100
Tetramisole 1 mM	98	94	101	95	116	97

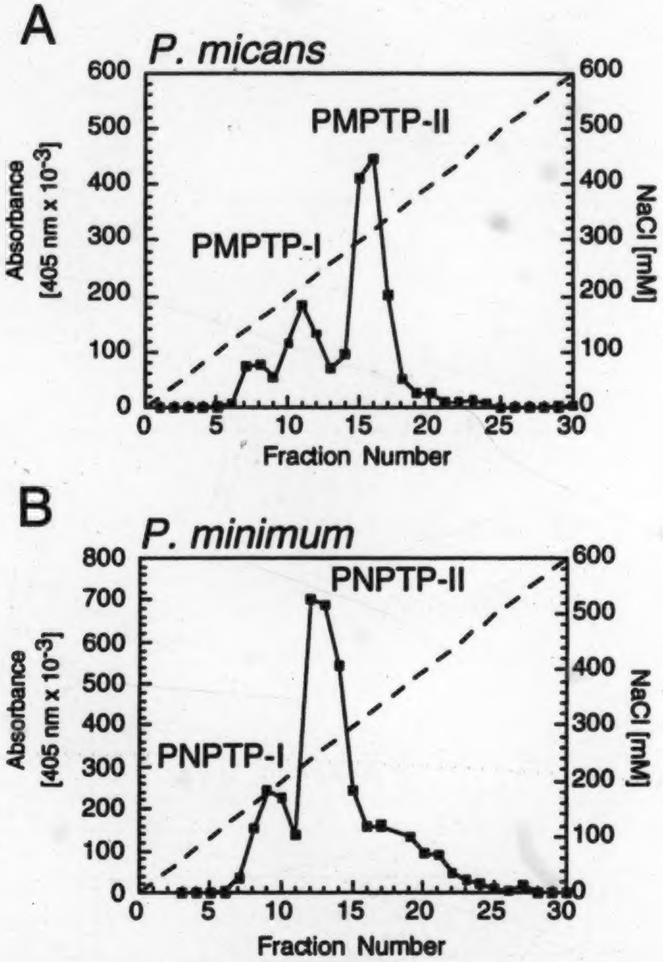


Fig. 3.5 Identification of protein tyrosine phosphatase activities in Mono-Q fractions of *Prorocentrum micans* (**A**) and *Prorocentrum minimum* (**B**) cell extracts. Aliquots (20 µL) of each fraction (1 mL) were assayed as delineated in the legend to Fig. 3.4. Three peaks of activity are seen in *P. micans*. The latter two major peaks were termed PMPTP-I and PMPTP-II. Two peaks of activity were seen in *P. minimum*, referred to as PNPTP-I and PNPTP-II according to their elution position.

Dephosphorylation of Phosphotyrosine Protein Substrates by PLPTP-I and PLPTP-II

Both PLPTP-I and II were analyzed for their ability to dephosphorylate polypeptide substrates containing labeled phosphotyrosine (Fig. 3.6A). PLPTP-I dephosphorylated myelin basic protein and angiotensin that had been phosphorylated by the protein tyrosine kinase p56^{lck} on tyrosine residues. PLPTP-II dephosphorylated phosphorylated myelin basic protein, but to a lesser degree than PLPTP-I, reflecting the lower activity of PLPTP-II in whole cell extracts. However, PLPTP-II did not show any significant activity toward isotopically phosphorylated angiotensin. It is possible that angiotensin is not an ideal substrate for PLPTP-II. Taken together with our pNPP assay results, it appears that the marine dinoflagellate *P. lima* possesses two major protein tyrosine phosphatase activities.

Both PLPTP-I and II were analyzed for their ability to dephosphorylate PLIK 1A which had autophosphorylated on tyrosine residues. Since boiling abolishes PLIK 1A binding to phosphocellulose, active PLIK 1A was necessary for efficient assay. Active radiolabelled PLIK 1A was therefore incubated with concentrated PLPTP-I or II preparations, resulting in an equilibrium of autophosphorylation and dephosphorylation. Both PLPTP-I and II dephosphorylated PLIK 1A. However, PLPTP-II produced an approximately 6-fold greater dephosphorylation of PLIK 1A in the 1 hour incubation relative to an equivalent amount of PLPTP-I preparation (Fig. 3.6B), based on densitometric analysis of the autoradiogram. This evidence suggests that PLPTP-II may be more specific for PLIK 1A than PLPTP-I, especially when we consider that identical volumes of the same enzyme preparations employed in Fig. 3.6B produced a 2.5-fold greater *p*NPPase activity of PLPTP-I over PLPTP-II. This further suggests that PLPTP-I and II represent two distinct PTPase activities.

Immunological Evidence for a cdc2-like Protein Kinase in *Prorocentrum lima.*

It has been observed that growth rates of certain dinoflagellates increase upon exposure to platelet derived growth factor and phorbol esters [36] and that a cdc2-like protein kinase is present in the dinoflagellate *Crypthecodinium cohnii* [35]. Taken together with the finding that both protein tyrosine kinases and phosphatases are present in *P. lima*, it was hypothesized that growth control in dinoflagellates is most likely similar to other unicellular eukaryotes. It was of interest, then, to determine if a cdc2-like protein kinase could be detected in *P. lima* where okadaic acid might affect signal transduction. A *P. lima* protein extract was separated by SDS-PAGE, transferred to nitrocellulose and

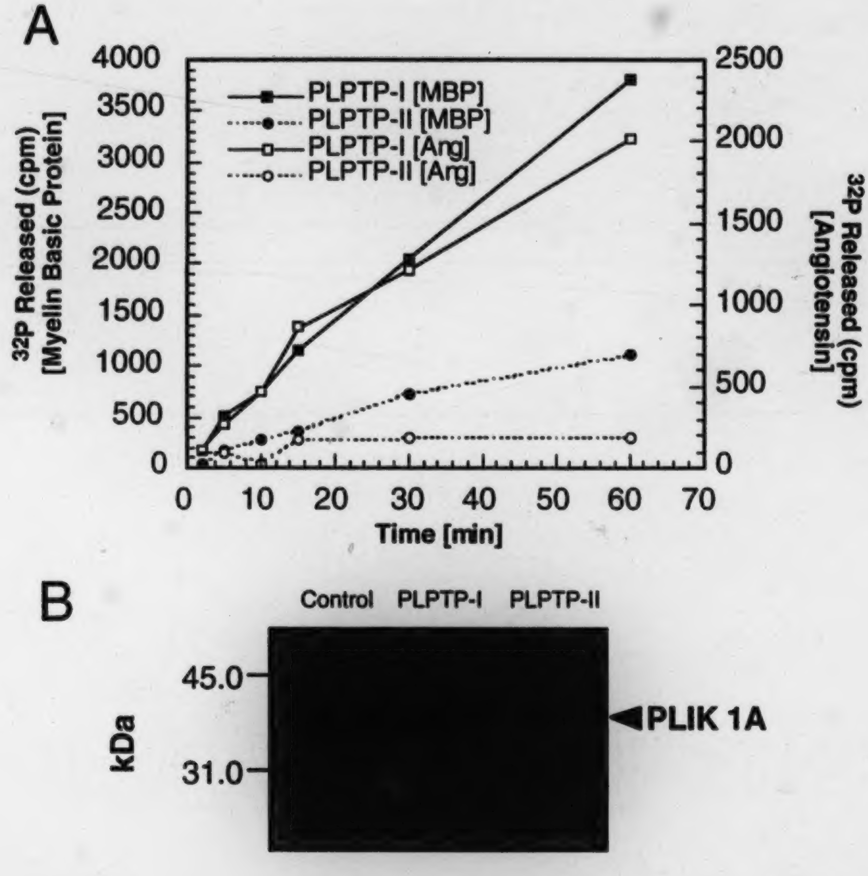


Fig. 3.6 Dephosphorylation of isotopically phosphorylated protein substrates by PLPTP-I and PLPTP-II. **A.** Isotopically tyrosine-phosphorylated myelin basic protein (MBP) or angiotensin was mixed with either PLPTP-I or PLPTP-II Mono-Q pools for indicated lengths of time and the reactions quenched upon the addition of acidic charcoal. The charcoal was pelleted by centrifugation and ^{32}P release was measured in the supernatant by Cherenkov counting. Only PLPTP-I was able to dephosphorylate both MBP and angiotensin; PLPTP-II demonstrated little activity toward angiotensin. **B.** Autoradiogram of PLIK 1A dephosphorylation by PLPTP-I and PLPTP-II. Isotopically autophosphorylated PLIK 1A was incubated with either buffer (control), PLPTP-I, or PLPTP-II for 1 hr at 30 °C, run on SDS-PAGE (12%), and an autoradiogram produced. Further experimental details are described in section 3.3. Both PLPTP-I and PLPTP-II were able to dephosphorylate PLIK 1A.

immunoblotted employing a monoclonal antibody directed against the PSTAIR peptide motif characteristic of the cyclin-dependent protein kinases that regulate the cell cycle (Fig 3.7). As a positive control, a cell extract from the fission yeast *Schizosaccharomyces pombe* was immunoblotted in parallel with the *P. lima* extract. Several proteins of varying molecular weight were detected in the *S. pombe* extract, including a strong band at 34 kDa, which corresponds to the molecular weight of cdc2 kinase. In the *P. lima* cell extract, a single protein band with an apparent molecular weight of 34 kDa was detected, strongly suggesting the presence of a cdc2-like protein kinase in *P. lima.*

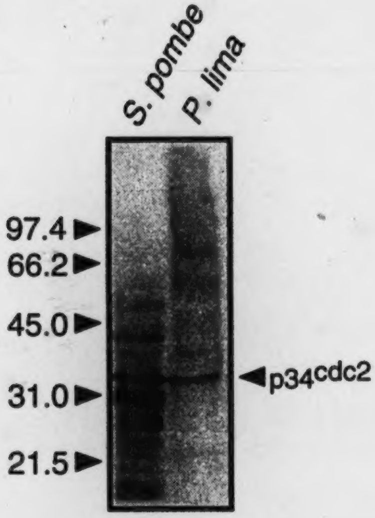


Fig. 3.7 Immunological detection of a cdc2-like protein kinase in *Prorocentrum lima*. A cell extract from the fission yeast *Schizosaccharomyces pombe* was run on SDS-PAGE (12%) alongside a cell extract from the toxic dinoflagellate *P. lima*, as described in section 3.3. The proteins were transferred to nitrocellulose and immunoblotted with a monoclonal antibody directed against the PSTAIR peptide motif of cdc2-cyclin dependent protein kinases (UBI, Lake Placid, NY). A strong band observed in the *S. pombe* extract running at about 34 kDa confirms the presence of cdc2 kinase in the fission yeast. A single major band observed in the *P. lima* cell extract also runs at approximately 34 kDa, suggesting the presence of a cdc2-like kinase in the toxic dinoflagellate.

3.5 ▪ DISCUSSION

To this point, I have provided evidence that the components required for reversible tyrosine protein phosphorylation, namely protein tyrosine kinases and protein tyrosine phosphatases, exist in the okadaic acid-producing marine dinoflagellate *Prorocentrum lima*. After yeast and trypanosomes, *P. lima* represents only the third class of unicellular eukaryotes to my knowledge reported to possess components of signaling involving reversible tyrosine protein phosphorylation. The evidence that PLIK 1A is a novel autophosphorylating protein tyrosine kinase represents the first direct identification of a protein tyrosine kinase activity in a marine dinoflagellate. The presence of phosphotyrosine was unambiguously established by phosphoamino acid analysis following acid hydrolysis of the protein and cross-reactivity with a phosphotyrosine antibody. Likewise, identification of PLPTP-I and II as novel protein tyrosine phosphatases in *P. lima* represents the first characterization of this class of enzymes in a marine dinoflagellate.

I was unable to stimulate PLIK 1A activity by chemical additions to the protein kinase assay, nor does PLIK 1A phosphorylate two peptides corresponding to the autophosphorylation sites of $pp60^{src}$ or $p56^{lck}$. In addition, PLIK 1A activity is not diminished by the general protein tyrosine kinase inhibitors herbimycin A and genistein.

A potential role for PLIK 1A in signal transduction may involve interaction with src homology-2 (SH-2) domain-containing proteins rather than direct phosphorylation of downstream effector substrates. Therefore, it will be of interest to characterize proteins that interact with PLIK 1A. Superdex-75 gel filtration analysis of active PLIK 1A-containing Mono-Q fractions resulted in two peaks of activity possessing apparent native molecular masses within the ranges of 47 to 55 kDa and 35 to 40 kDa. The smaller Superdex-75 peak corresponds well with the 38 kDa molecular mass observed in autoradiograms of Mono-Q fractions containing PLIK 1A. The absence of an autophosphorylated higher molecular mass protein in autoradiograms suggests that a small molecular mass polypeptide associates with PLIK 1A in *P. lima* cell extracts.

Two peaks of protein tyrosine phosphatase activity, referred to here as PLPTP-I and II, were identified using the general phosphatase substrate *p*NPP. Both activities were inhibited by the classical protein tyrosine phosphatase inhibitors molybdate and zinc. Neither activity was inhibited by tartrate, an inhibitor of some acid phosphatases, tetramisole, an inhibitor of alkaline phosphatases, nor microcystin LR, a potent inhibitor of type-1 and -2A protein serine/threonine phosphatases. In addition, both PLPTP-I and

II were capable of dephosphorylating myelin basic protein isotopically labeled on tyrosine while only PLPTP-I dephosphorylated isotopically phosphorylated angiotensin. These experiments, coupled with the ability of both PLPTP-I and II to dephosphorylate PLIK 1A (see also below), suggest that PLPTP-I and II are not acid or alkaline phosphatases but *bona fide* protein tyrosine phosphatases.

Gel filtration analysis resulted in an apparent native molecular mass range of 150 to 168 kDa for PLPTP-I and two peaks within 150 to 168 kDa and 73 to 82 kDa for PLPTP-II. The earlier-eluting peak contained in PLPTP-II Mono-Q fractions is inhibited in a similar manner to that of the PLPTP-I Mono-Q fractions and thus most likely represents contamination of the PLPTP-II pool by PLPTP-I enzyme. Differential inhibition by molybdate of the two PLPTP-II gel filtration peaks and different substrate specificities between PLPTP-I and II suggest that they represent two distinct protein tyrosine phosphatase activities in *P. lima* and not monomers and dimers of the same phosphatase catalytic subunit as their apparent molecular masses might suggest. In the cases of radiolabelled MBP and angiotensin, PLPTP-I more efficiently dephosphorylated the substrate. Conversely, PLPTP-II dephosphorylated PLIK 1A more efficiently than PLPTP-I. The ability of PLPTP-I and II to dephosphorylate tyrosine-phosphorylated PLIK 1A represents the first identification of an endogenous protein phosphatase substrate in a marine dinoflagellate.

P. lima is a primary producer of the potent protein serine/threonine phosphatase inhibitor okadaic acid. Therefore, it was important to establish whether two closely related Prorocentrale species that do not produce okadaic acid, *P. micans* and *P. minimum*, possessed PTPase activities. Since both species possessed detectable PTPase activities, there is no evidence to suggest that okadaic acid regulates PTPase activity in marine dinoflagellates.

One of the hallmarks of eukaryotic cells is the possession of a cell cycle with discrete phases of growth, DNA synthesis, and mitosis. Signaling molecules including cyclins and cyclin-dependent protein kinases, MAP kinase cascades and protein tyrosine phosphatases play major roles in the regulation of the eukaryotic cell cycle [44]. Our data indicate that the okadaic acid-producing marine dinoflagellate *Prorocentrum lima* possesses molecules governing reversible tyrosine protein phosphorylation, namely protein tyrosine kinases and protein tyrosine phosphatases. In addition, it has been shown that another dinoflagellate species possesses a cdc2-like protein kinase activity [35] and that dinoflagellate growth is accelerated in response to a mammalian growth factor or phorbol esters [36]. Immunoblot analysis of a *P. lima* cell extract employing cdc2 monoclonal antibodies clearly identified a 34 kDa protein, likely corresponding to a cdc2-

like protein kinase and suggesting that *P. lima* may also possess a cdc2 protein kinase. Taken together, this evidence suggests that marine dinoflagellates possess cell cycle control signaling mechanisms similar to those of higher eukaryotes and yeast. This further supports placing marine dinoflagellates within the eukaryotic organization, despite their lack of histones and nucleosome structure. It will be of interest to determine if marine dinoflagellates utilize similar signaling strategies to yeast, namely the release of chemical factors into their environment to communicate their growth status that stimulate signaling molecule cascades. The data presented in this chapter clearly suggest that the marine dinoflagellates represent a system in which classical pathways of signal transduction may be studied in a marine organism.

Addendum

Recent sequencing and analysis of the entire genome of the budding yeast *Saccharomyces cerevisiae* has lead to the observation that there exist no open reading frames that possess hallmark PTK coding sequences in this unicellular organism [45]. This seems paradoxical when we consider that phosphotyrosine has been detected in this species and is a vital part of its cell cycle control mechanisms. However, closer examination of the *S. cerevisiae* genome revealed the presence of dual specificity protein kinases that may account for the presence of phosphotyrosine. It was hypothesized that the development of dedicated protein tyrosine kinases coincides with the emergence of metazoans as a means to coordinate multicellular growth and development. It is of note, therefore, that attempts to isolate dedicated PTK cDNAs from *P. lima* employing degenerate PCR primers corresponding to hallmark PTK sequences (a technique that has been highly successful with metazoan DNA) [46], failed to generate any PCR product. Likewise, screening a *P. lima* cDNA library with degenerate oligonucleotides patterned after hallmark PTK sequences failed to yield any positive clones. This suggests that, like the budding yeast, there exist no dedicated PTKs in *P. lima*. Therefore, since PLIK 1A does phosphorylate tyrosine residues, it is most likely a dual-specificity kinase. The hypothesis that PLIK 1A is a dual-specificity kinase is supported by our observations that it is unable to phosphorylate substrates targeted by dedicated protein tyrosine kinases and its activity is not affected by the general protein tyrosine kinase inhibitors genistein and herbimycin A. This evidence further supports the hypothesis that dedicated PTKs evolved with the emergence of metazoans and demonstrates that marine dinoflagellates contain phosphotyrosine signaling and cell cycle control mechanisms that are characteristic of unicellular eukaryotes.

3.6 ■ PART II: PROTEIN SERINE/THREONINE KINASES IN MARINE DINOFLAGELLATES

The level of protein phosphorylation on serine and threonine residues in eukaryotic cells is regulated by the opposing actions of protein serine/threonine kinases and phosphatases. cAMP-dependent protein kinase (PKA) has been extensively studied as a model for the entire protein kinase family due to its well defined structure and relative ease of purification and assay [47-49].

Mammalian forms of PKA are tetramers consisting of two regulatory (R) subunits and two catalytic (C) subunits. There exist two distinct types of R subunits: the R_I subunit is smaller than the R_{II} (48 kDa and 55 kDa, respectively) and the R_{II} subunit is autophosphorylated on Ser-95 [50]. This site can be dephosphorylated by calcineurin (PP-2B) [51]. The binding of cAMP to specific sites on the R subunit leads to conformational changes that liberate active C subunit from the inhibitory R dimer. The free C subunit phosphorylates target proteins in the cytoplasm and nucleus, regulating a number of cellular effects [52-55]. Among these are regulation of glycogen synthase activity [56], regulation of protein phosphatase activity through phosphorylation and activation of protein phosphatase inhibitor-1 [43], and regulation of transcription through activation of the cAMP-responsive element binding transcription factor (CREB) [57].

Thus far, PKA has not been identified in higher plants [58]. However, a number of lower eukaryotes are known to possess PKA, including the photosynthetic algal flagellate *Euglena gracilis* [59]. In addition, the genome of the budding yeast *Saccharomyces cerevisiae* encodes three C subunits [60]. The *Dictyostelium* PKA isoform is believed to be an RC dimer based on a lack of the R-R dimerization region in the R subunit gene of that organism [61]. The cytoplasmic enzyme in *Paramecium* was characterized as an RC dimer by gel filtration analysis [62], although the form of the enzyme isolated from cilia is an R_2C_2 tetramer [63]. This evidence has lead investigators to hypothesize that the R_2C_2 tetramer found in higher eukaryotes evolved from a simpler RC dimer in lower eukaryotes.

One group of organisms that may shed light on the evolution of PKA and PKA-signaling are the marine dinoflagellates. In accordance with my objective to investigate the nature of signal transduction among marine dinoflagellates, I endeavoured to identify and characterize PKA in marine dinoflagellates with particular attention given to the *P. lima* system in which okadaic acid is produced and may interfere with signal transduction. In part two of this chapter, evidence for PKA activity in *P. lima, P. micans*, and *P. minimum* is presented. The *P. lima* enzyme was characterized via anion exchange and gel filtration chromatography and appears to consist of two different isoforms in the R_2C_2 configuration. Whole cell extracts of *P. micans* and *P. minimum* treated with the specific PKA inhibitor peptide PKI (5-24) or cAMP demonstrated altered intensities of phosphopeptide [^{32}P]-labeling suggesting regulation of a protein phosphatase activity via PKA activity. The presence of PKA in marine dinoflagellates further strengthens the hypothesis that marine dinoflagellates possess advanced signal transduction mechanisms paralleling those of eukaryotic organisms.

3.7 ■ MATERIALS AND METHODS

Preparation of Whole Cell Extracts

Marine dinoflagellate cultures were harvested by centrifugation at 1250 *g* (r_{av}=18.4 cm). Extraction buffer (4 mL) comprising 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1% Brij 35 (v/v), 0.1 mM EDTA, 0.1 mM EGTA, and a cocktail of protease inhibitors (described in [64]) were added per gram of wet weight cell pellet. Cell suspensions were then passed through a French press twice at 16000 psi. Resulting homogenates were then centrifuged briefly at 200 *g* (r_{av}=18.4 cm) at 4 °C. Resulting supernatants were then centrifuged more extensively at 16000 *g* (r_{av}=7.3 cm) for 20 min at 4 °C. Supernatants were then filtered through Millipore GS filters (0.22 μm) and used immediately or flash frozen and stored at -70 °C.

Protein Kinase Assays Employing Mono-Q Anion Exchange Fractions

Marine dinoflagellate extracts were fractionated utilizing a Mono-Q anion exchange column developed with a linear gradient from 0 to 1M NaCl in running buffer (20 mM triethanolamine-HCl, pH 7.0, 10% glycerol (v/v), 0.1 mM EGTA, 25 μM DTT, and 25 μM PMSF). An aliquot (40 μL) of Mono-Q fractions (1 mL) was incubated in reactions (total volume 46 μL) containing 25 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 6.67 μM [γ-^{32}P] ATP (specific activity of 3000 Ci / mmol), and either 10 μg casein or 10 μg mixed histones and incubated at 30 °C for 60 min. Reactions were quenched by spotting 40 μL of the reaction onto P-81 phosphocellulose paper, which were washed three times in 0.75 M H_3PO_4 for 5 min and counted using a scintillation counter (Pharmacia). Alternatively, reactions were stopped by the addition of Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 20% SDS (w/v), 5% β-mercaptoethanol (v/v), 0.00625% bromophenol blue (w/v)) followed by incubation at 85 °C for 10 minutes. Samples were then loaded and run on SDS-PAGE (15%), dried on a Biorad Gel Drier and exposed to X-ray film (Kodak) to produce an autoradiogram.

To detect cAMP-dependent protein kinase activity, aliquots (35 µL) of Mono-Q fractions (1 mL) were mixed with a substrate/agonist mixture (10 µL) consisting of 0.5 mM Kemptide in the presence or absence of 125 µM cAMP. Kinase reactions were initiated with the addition of a kinase reaction mixture (5 µL) containing 250 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 6.67 µM [γ-^{32}P] ATP (specific activity of 3000 Ci / mmol) at 30 °C. Reactions were quenched after 60 min by spotting 45 µL of the reaction onto P-81 phosphocellulose paper (Whatman). Phosphocellulose filters were then treated as described above.

Superdex Gel Filtration Chromatography

Active Mono-Q fractions were rechromatographed on a Superdex-200 gel filtration column with 50 mM Tris-HCl pH 7.5 , containing 100 mM NaCl, 0.1 mM EGTA, 25 µM DTT, and 25 µM PMSF. Aliquots (35 µL) of fractions (200 µL) were then assayed for kinase activity as described above. Molecular weight standards (13.7 kDa to 669 kDa) were from Pharmacia.

In vitro Whole Cell Extract Phosphorylation Assays

Marine dinoflagellate extracts (20 µL) were incubated for 2.5 hours at 30 °C with Tris-HCl, pH 7.5 and [γ-^{32}P] ATP at final concentrations of 20 mM and 1.07 µM (specific activity 3000 Ci / mmol) respectively, and the appropriate agonist/inhibitor in a final volume of 25 µL. The reaction was stopped by the addition of Laemmli buffer followed by incubation at 85 °C for 10 minutes. Samples were then loaded and run on SDS-PAGE (12%), dried on a Biorad Gel Drier and exposed to X-ray film (Kodak) for various lengths of time to produce an autoradiogram. Prestained molecular weight standards (Biorad) were employed to calculate molecular weights.

3.8 ■ RESULTS

Identification of Casein and Histone Kinases in *Prorocentrum lima*

Fractions of *P. lima* whole cell lysates produced employing a French press and separated by Mono-Q anion exchange chromatography were assayed for their ability to phosphorylate casein or mixed histones, two common protein serine/threonine kinase substrates (Fig. 3.8). Two peaks of kinase activity were identified. The first peak of

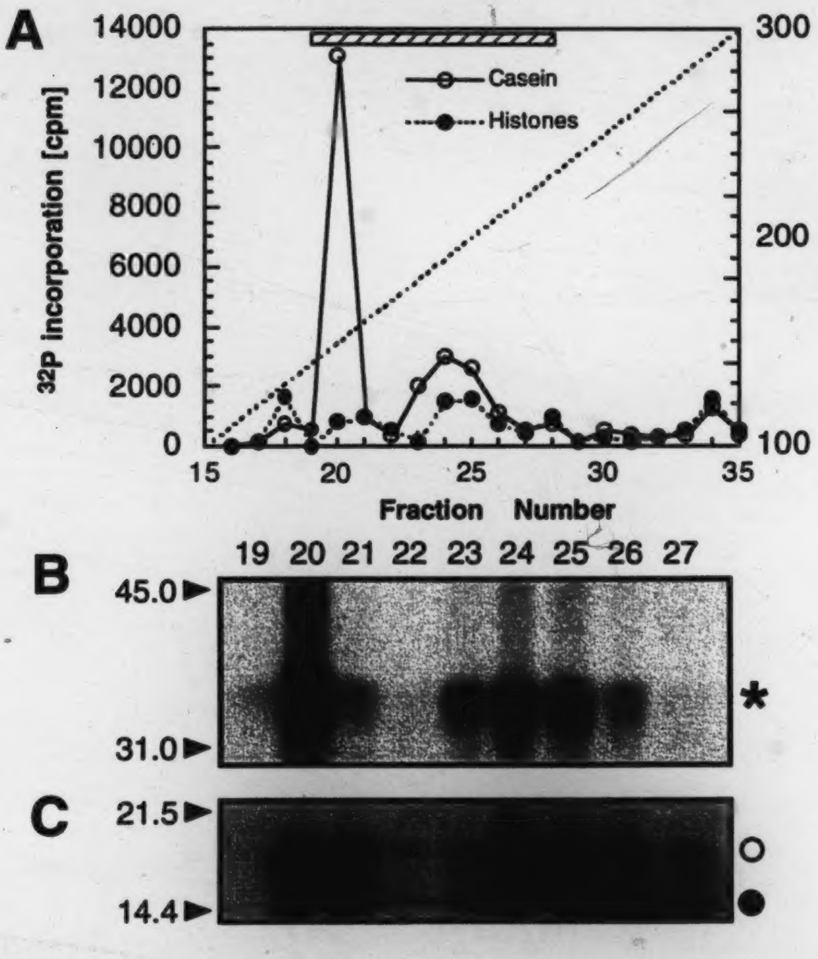


Fig. 3.8 Identification of casein and histone kinases in *Prorocentrum lima*. A. Aliquots (40 µL) of Mono-Q fractions (1 mL) of a *P. lima* cell extract produced with a french press pressure cell were incubated with either casein (10 µg) or mixed histones (10 µg) in the presence of $[\gamma\text{-}^{32}P]$ ATP for 1 hr at 30 °C. Reactions were then filtered through phosphocellulose, washed and the amount of radiolabel incorporated measured on a scintillation counter. A major peak of casein phosphorylating activity was observed in fraction 20, with a minor peak in fractions 23 to 25. These fractions contained substantially less histone phosphorylating activity. The fractions indicated by the hatched bar were further analyzed by SDS-PAGE (15%) and autoradiography (panels B and C). B. Autoradiogram of reaction mixtures described above containing casein. The position of α-casein is indicated on the right with an asterisk. A significant amount of phosphate incorporation was observed in fraction 20, while less incorporation was seen in fractions 23 to 25, correlating with scintillation measurements presented in A. C. Autoradiogram of reaction mixtures described above containing histones. The position of histones is indicated on the right by open and closed circles. Two peaks of activity were observed. The first was in fractions 20 and 21 where the higher molecular mass histone indicated by the open circle seemed to be a preferred substrate. Alternatively, the second peak of activity in fractions 24 to 26 appeared to preferentially phosphorylate the lower molecular mass histone as substrate, indicated by the closed circle.

activity efficiently phosphorylated the α subunit of casein and preferred the higher molecular weight histone as a substrate. Likewise, the second peak of activity was able to phosphorylate the α subunit of casein, but preferred the smaller molecular weight histone as an exogenous substrate. As casein and histones are common targets for several protein serine/threonine kinases, the results of this experiment were taken as a positive indication that marine dinoflagellates possess protein serine/threonine kinase activity.

The results of these experiments were also encouraging as they demonstrated that marine dinoflagellate cell lysates produced using a French press contained detectable protein kinase activity. Therefore, I employed the French press when producing cell extracts to identify and characterize PKA activity in marine dinoflagellates.

Identification and Characterization of cAMP-dependent Protein Kinase in *Prorocentrum lima*

Mono-Q anion exchange chromatography fractions of marine dinoflagellate whole cell extracts were assayed for their ability to phosphorylate Kemptide (Fig. 3.9A). Two cAMP-stimulated peaks of activity were identified in *P. lima* cell extracts. This suggests the presence of two different regulatory subunits in marine dinoflagellates, a situation which would parallel the mammalian case [50]. Therefore, the first peak of Kemptide phosphorylating activity is referred to as PLPKA-I and the second is termed PLPKA-II.

Employing a peptide containing a classical PKA phosphorylation sequence from *P. lima* PP-1 (PLPP-1) as substrate (NRIYGFYDECKRRYSIRLWKTSTD), two cAMP-stimulated peaks of activity corresponding to PLPKA-I and II were identified (Fig. 3.9B). An additional peak of activity was observed, eluting at lower salt concentrations. This peak is inhibited by the presence of 25 μM cAMP in the kinase assay. This evidence suggests that this peak represents free catalytic subunits of a *P. lima* PKA-like activity, as control experiments demonstrated that bovine heart PKA catalytic subunit manifests lower phosphorylating activity in the presence of 25 μM cAMP in our assay, most likely due to the presence of AMP in the aliquots of cAMP employed which acts as an inhibitor of kinase activity by occupying the ATP-binding pocket of the kinase. Therefore, this peak is referred to as the PLPKA catalytic subunit. Surprisingly, no detectable PKA activity was found in either *P. micans* or *P. minimum* Mono-Q anion exchange chromatography fractions following our protocol.

The ability of PLPKA-II to phosphorylate Kemptide was analyzed employing the capillary electrophoresis-based assay described in Chapter 2 (Fig. 3.10). Under these conditions, a peak of phosphorylated Kemptide is readily detected and was verified by

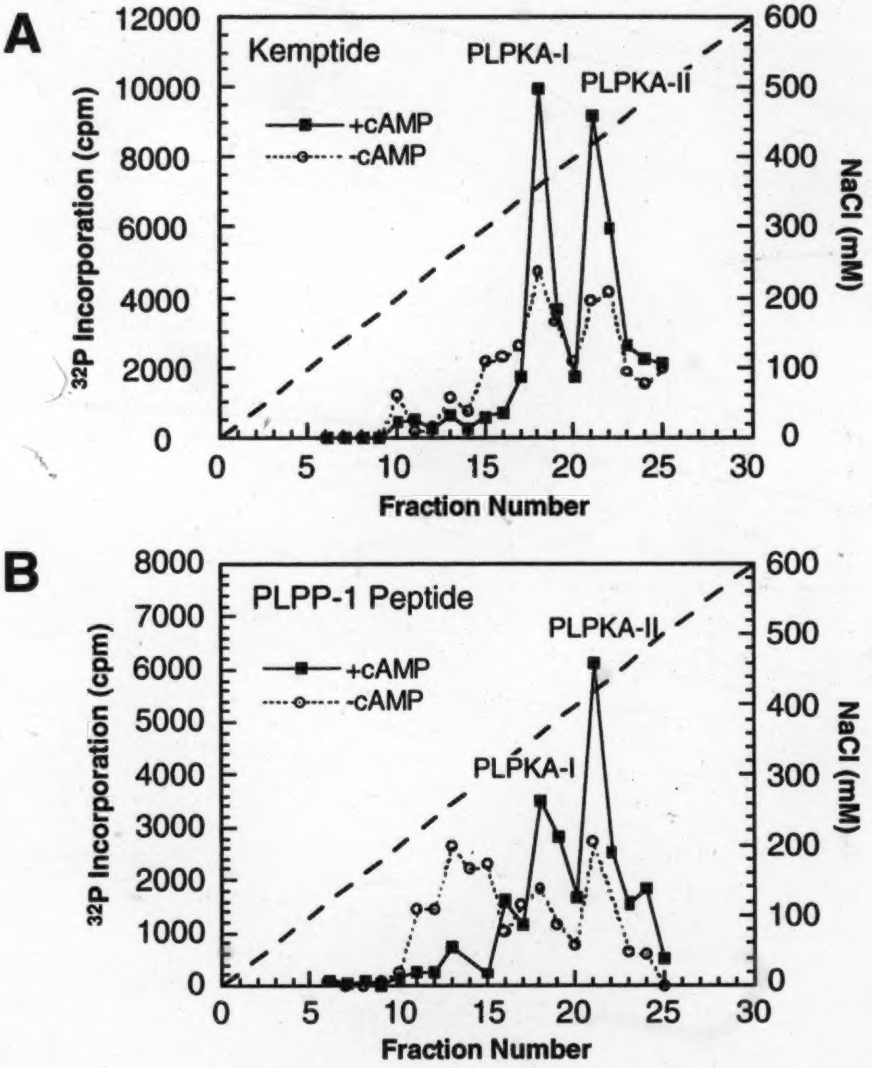


Fig. 3.9 Identification of PKA isoforms in Mono-Q fractions of a *Prorocentrum lima* cell extract employing Kemptide (**A**) or a fragment of PP-1 from *P. lima* (referred to as PLPP-1 peptide) (**B**). Aliquots (35 μL) of fractions (1 mL) of a *P. lima* extract subjected to Mono-Q anion exchange chromatography were incubated with the PKA substrate peptide Kemptide or the PLPP-1 peptide in the presence of [γ-^{32}P]ATP. Two peaks of protein kinase activity stimulated by cAMP were identified, referred to as PLPKA-I and -II by their elution position off Mono-Q. An earlier-eluting peak of activity inhibited by cAMP is observed when the PLPP-1 peptide is employed as substrate. This peak of activity is referred to as PLPKA catalytic subunit for further characterization.

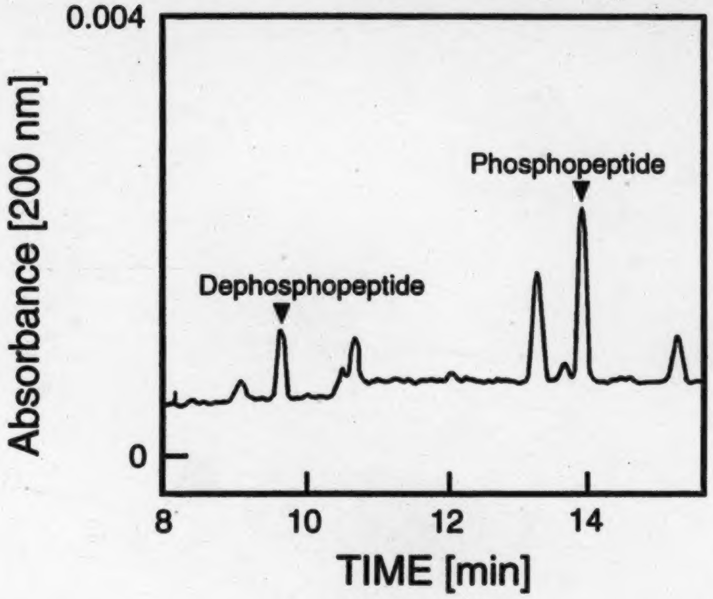


Fig. 3.10 Detection of cAMP-dependent protein kinase activity in *Prorocentrum lima* employing a capillary electrophoresis-based assay. An aliquot (35 μL) corresponding to Mono-Q fraction 18 (1 mL, see Fig. 3.9) of a *P. lima* cell extract was incubated with the peptide substrate Kemptide in the presence of cold ATP, magnesium, and cAMP and incubated for 1 hr at 30 °C. Reactions were then analyzed employing the capillary electrophoresis protocol described in Chapter 2 at pH 2.0. The total amount of peptide applied to the capillary was 0.5 pmol. A peak of phosphopeptide was detected and verified by spiking the sample with phosphorylated Kemptide. Other peaks were also detected and present only when Kemptide was included in the reactions, suggesting that they may represent proteolytic fragments of the peptide kinase substrate.

spiking the sample with phosphopeptide standard. Control experiments demonstrated that the formation of a phosphopeptide peak was dependent on the presence of exogenous ATP and Mg^{2+}. Additional control experiments in which only the PLPKA-II fraction was analyzed by capillary electrophoresis revealed the presence of a small peak eluting just after 10 min. This peak is also present in the experiment described in Fig. 3.10, in addition to several other peaks. It is hypothesized that these additional peaks represent the products of proteolysis of the Kemptide substrate added to the marine dinoflagellate Mono-Q fractions. As discussed later, proteolysis was a constant concern when handling marine dinoflagellate cell extracts and fractions.

Active Mono-Q peaks were pooled and analyzed by Superdex-200 gel filtration chromatography (Fig. 3.11). One peak of activity was observed in the PLPKA catalytic subunit pool. This peak (elution time of approximately 155 minutes) coincided very

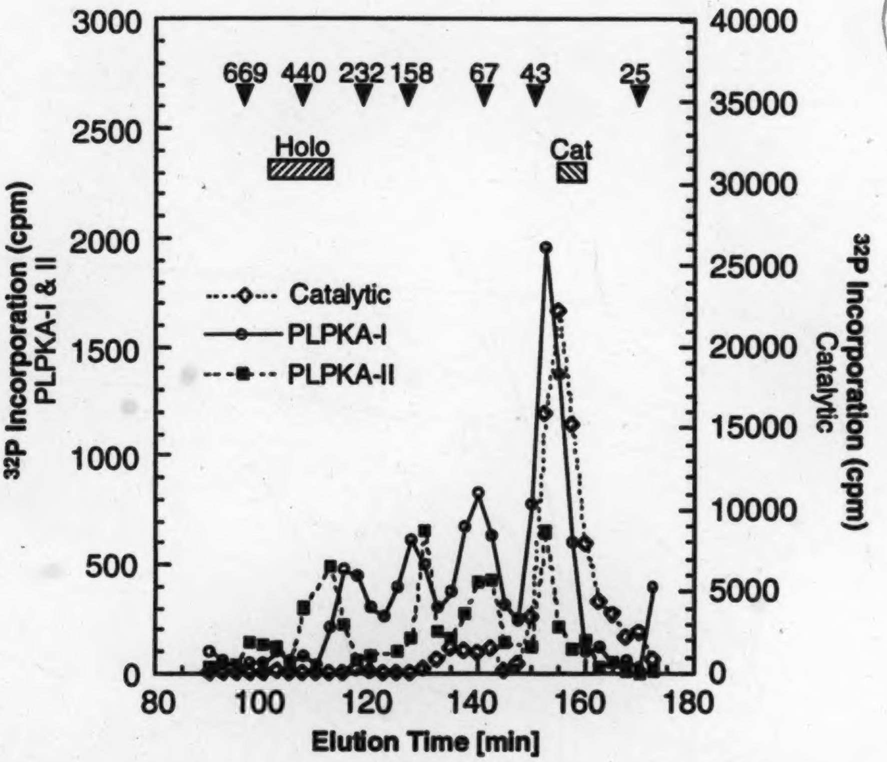


Fig. 3.11 Futher characterization of *Prorocentrum lima* cAMP-dependent protein kinase activities described in Fig. 3.9 by Superdex-200 gel filtration chromatography. The elution positions of molecular weight markers (669 to 25 kDa) are shown across the top. The elution positions of bovine heart PKA catalytic subunit (Cat) and holoenzyme (Holo) are shown as hatched bars. PLPKA catalytic subunit from Mono-Q eluted in the same position as bovine PKA free catalytic subunit. Both PLPKA-I and -II demonstrated four peaks of Kemptide-phosphorylating activity. Of these, the peaks representing the largest molecular weights eluted in the same position as bovine PKA holoenzyme. Those representing the smallest molecular weights eluted in the same position as bovine PKA free catalytic subunit. PLPKA-II possessed a larger holoenzyme size than PLPKA-I.

closely with the elution time of the free catalytic subunit of bovine heart PKA. This peak of activity was observed in both the PLPKA-I and II pools, as would be expected from a PKA-like activity. Three additional peaks of Superdex-200-resolvable activity were identified in the PLPKA-I and II pools. Of these, the peak representing the largest molecular weight eluted slightly after that of bovine heart PKA holoenzyme, suggesting that the *P. lima* PLPKA holoenzymes are smaller than that from bovine heart. In this regard, the PLPKA-II holoenzyme is slightly larger than the PLPKA-I holoenzyme. This supports the identification of PLPKA-I and II as homologs of mammalian PKA complexes containing different regulatory subunits, since the mammalian R_{II} subunit is larger than the R_I [50]. The two peaks between the holoenzyme and the free catalytic subunit may represent complexes of different combinations of regulatory and catalytic subunits.

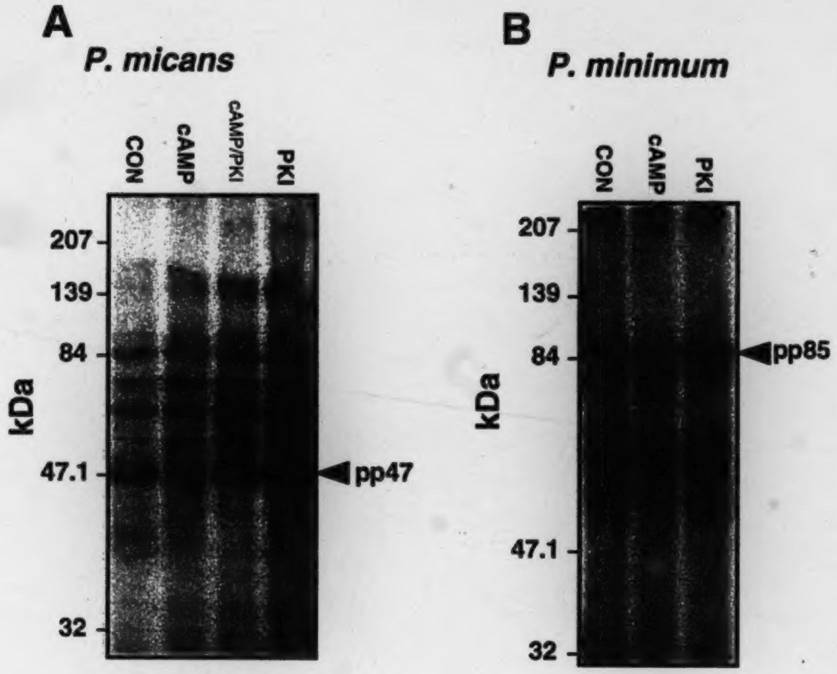
Identification of cAMP-dependent Protein Kinase in *Prorocentrum micans* and *Prorocentrum minimum*

In order to identify potential PKA substrates in marine dinoflagellates, we incubated whole cell extracts of *P. micans* and *P. minimum* with [γ-^{32}P] ATP and analyzed the pattern of phosphopeptides by SDS-PAGE under various conditions. Unfortunately the presence of high concentrations of okadaic acid in *P. lima* caused analysis of phosphopeptides in these cell extracts to be difficult.

In *P. micans* cell extracts, a 47 kDa protein, referred to as pp47, was hyperphosphorylated in the presence of the specific PKA inhibitor PKI and was not phosphorylated in the presence of the PKA agonist cAMP (Fig. 3.12A). Due to the specific nature of PKI and the agonistic effect of cAMP, this evidence suggests that PKA is present in *P. micans* despite our negative results employing Mono-Q anion exchange fractions of whole cell extracts. These results also suggest that PKA affects the phosphorylation state of pp47 indirectly.

In *P. minimum* cell extracts, an 85 kDa phosphoprotein called pp85 responded to PKA inhibitor and agonist in the same manner as pp47 of *P. micans* (Fig. 3.12B). As with *P. micans*, this evidence suggests that PKA activity is also present in *P. minimum*.

We envision that the simplest mode of action of PKA with regard to the phosphorylation states of pp47 and pp85 would be through direct regulation of a protein phosphatase. When PKA activity is stimulated by the addition of cAMP, PKA might phosphorylate and activate a protein phosphatase which then dephosphorylates pp47 or pp85 directly. Conversely, when PKA is inhibited by PKI, the protein phosphatase may



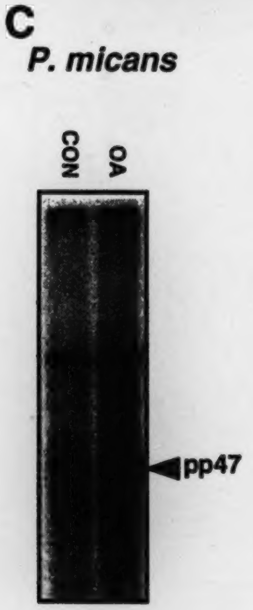


Fig. 3.12 Phosphopeptide analysis of *P. micans* and *P. minimum* cell extracts. Whole cell extracts (20 μL) of *P. micans* or *P. minimum* were incubated with [γ-^{32}P]ATP in the absence or presence of 25 μM cAMP and/or 4.4 μM PKI and the pattern of phosphopeptides analyzed by SDS-PAGE (15% panels A and C and 12% panel B). pp47 from *P. micans* (A) and pp85 from *P. minimum* (B) were hyperphosphorylated in the presence of the PKI and hypophosphorylated in the presence of cAMP relative to control reactions with no chemical additions (CON). pp47 was also hyperphosphorylated in the presence of 2 μM okadaic acid (OA) (C). These data suggest that PKA is regulating a protein phosphatase activity,which might be PP-1 or PP-2A.

be inactivated causing pp47 and pp85 to become hyperphosphorylated by an unknown (possibly constitutive) protein kinase activity.

This model suggests that pp47 and pp85 are protein phosphatase substrates. When okadaic acid was included in our *in vitro* phosphoprotein assay pp47 showed a slight increase in its phosphorylation state (Fig. 3.12C). The high concentration of okadaic acid employed prohibited differentiation between PP-1 and PP-2A activities.

3.9 ■ DISCUSSION

Marine dinoflagellate cell extracts chromatographed on Mono-Q anion exchange chromatography possessed two resolvable peaks of kinase activity that were capable of phosphorylating the general protein serine/threonine kinase substrates casein and mixed histones. This suggested that marine dinoflagellate cell extracts prepared with the use of a French press contain detectable protein kinase activity and thus the French press was employed to prepare cell extracts utilized to identify and characterize PKA in Prorocentrale species.

Three Mono-Q-resolvable peaks of protein kinase activity from *Prorocentrum lima* whole cell extracts that possess properties characteristic of cAMP-dependent protein kinase (PKA) were identified. The first Mono-Q peak coeluted with bovine heart PKA catalytic subunit on Superdex-200 gel filtration and PKA activity was inhibited by the addition of 25 μM cAMP. This behaviour was similar to that of the free PKA catalytic subunit from bovine heart in our assay. I postulate that this first Mono-Q peak represents free catalytic subunit of the *P. lima* homolog of PKA.

The two other peaks of Mono-Q activity may represent *P. lima* homologs of PKA holoenzyme. Both are stimulated by the addition of cAMP. Their different elution positions when separated by Mono-Q anion exchange chromatography is most likely due to different regulatory subunit composition. This is analogous to the mammalian case where PKA holoenzyme containing the R_I regulatory subunit elutes before that possessing the R_{II} regulatory subunit off anion exchange columns. Hence these two activities are referred to as PLPKA-I and II after the mammalian nomenclature. Superdex-200 gel filtration analysis of both PLPKA-I and II indicated that both *P. lima* PKA homologs are slightly smaller than the bovine heart PKA holoenzyme and that the PLPKA-II holoenzyme is slightly larger than the PLPKA-I holoenzyme. This is in accordance with the larger molecular weight of the mammalian R_{II} subunit.

Interestingly, no PKA activity was observed in Mono-Q anion exchange chromatography fractions of whole cell extracts of *P. micans* or *P. minimum*. However, phosphoprotein analyses of whole cell extracts under different conditions suggest the presence of PKA activity in *P. micans* and *P. minimum* since different levels of phosphorylation of specific proteins in the presence of the specific PKA inhibitor PKI and agonist cAMP were observed. One fundamental difference between *P. lima*, *P. micans* and *P. minimum* is that *P. lima* is an okadaic acid producer while the others are not. It is therefore possible that okadaic acid plays a positive role in regulating PKA activity or transcription/translation through an unknown mechanism. Another less likely possibility is that okadaic acid affects certain proteases such that in cell extracts PKA activity is not degraded as quickly as in *P. micans* or *P. minimum*.

Enzyme stability proved to be a constant problem during marine dinoflagellate PKA preparation from whole cell extracts and in Mono-Q fractions. A drastic decline in PKA activity was consistently observed within a day of cell extract preparation despite the presence of a powerful protease inhibitor cocktail in the cell extract. The chromatographic separation of free catalytic subunit from other combinations of regulatory and catalytic subunit complexes during gel filtration of holoenzyme preparations from Mono-Q anion exchange chromatography attests to the instability of the holoenzyme during our purification procedure. Despite preparing samples for gel filtration as quickly as possible and running the gel filtration chromatography step as soon as possible (often the same day as the Mono-Q anion exchange column), dramatic loss of protein kinase activity was observed, most notably with the PLPKA-II sample. Given that preparations of *P. lima* PP-1 do not degrade as rapidly, it is possible that there exists a protease in the marine dinoflagellate cell extract that is a) specific for a motif within the marine dinoflagellate PKA and b) not effectively inhibited by the panel of protease inhibitors employed.

Despite these obstacles, I was able to observe phosphoproteins in *P. micans* and *P. minimum* that possessed altered phosphorylation states upon the addition of PKA inhibitor or agonist. The effect of inhibiting PKA activity by the addition of PKI was hyperphosphorylation of pp47 from *P. micans* and pp85 from *P. minimum*. Conversely, the effect of stimulating PKA activity through the addition of cAMP was to decrease the phosphorylation states of pp47 and pp85. Although these effects are contrary to the concept that PKA directly acts on pp47 or pp85, they are striking enough to support the idea that PKA is indeed present in the non-okadaic acid producing marine dinoflagellates *P. micans* and *P. minimum*.

The effects of PKA inhibitor and agonist on pp47 and pp85 suggest that PKA may be regulating a protein phosphatase activity which may act directly upon pp47 or pp85. Addition of okadaic acid to *P. micans* cell extracts demonstrated an increase in the phosphorylation state of pp47, thereby suggesting that either PP-1 or PP-2A may be dephosphorylating these phosphoprotein substrates. Preliminary experiments employing the active fragment of inhibitor-1, a specific inhibitor of PP-1, suggested that PP-1 may be the protein phosphatase in question.

Taken together, the identification of phosphotyrosine signaling and protein serine/threonine phosphatases in Prorocentrale marine dinoflagellates has provided a clear indication of the nature of signal transduction in marine dinoflagellates. The evidence suggests that marine dinoflagellates possess signal transduction mechanisms similar to those utilized by eukaryotes (Fig. 3.13). This evidence clearly supports the hypothesis that marine dinoflagellates are true eukaryotes that have secondarily lost a gene group encoding histone proteins [15]. As such, the mesokaryotic hypothesis appears to be false and the classic phylogenetic position of marine dinoflagellates should be re-evaluated.

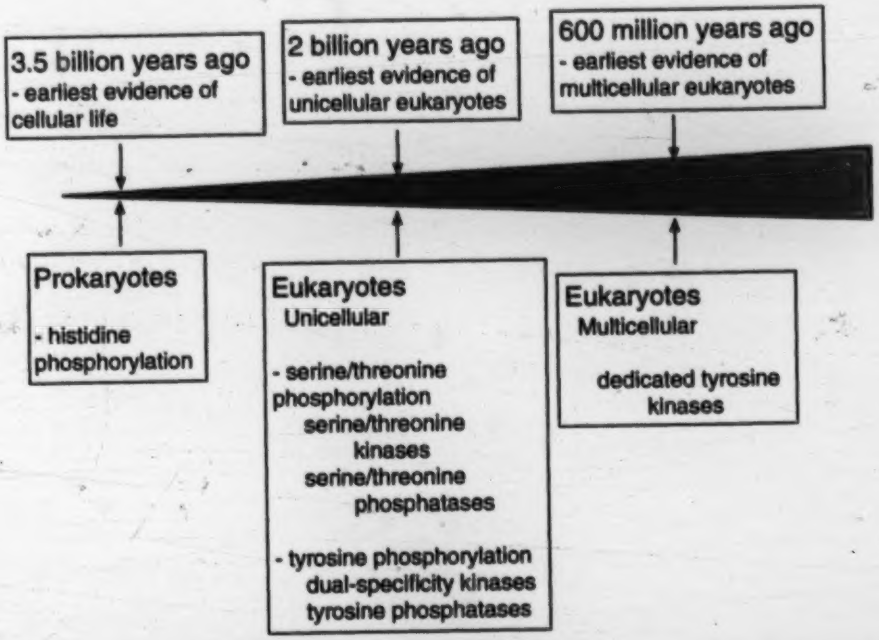

3.5 billion years ago
- earliest evidence of cellular life
2 billion years ago
- earliest evidence of unicellular eukaryotes
600 million years ago
- earliest evidence of multicellular eukaryotes
Prokaryotes
- histidine phosphorylation
Eukaryotes
Unicellular
- serine/threonine phosphorylation
serine/threonine kinases
serine/threonine phosphatases
- tyrosine phosphorylation
dual-specificity kinases
tyrosine phosphatases
Eukaryotes
Multicellular
dedicated tyrosine kinases

Fig. 3.13 Development of reversible protein phosphorylation through geological time. The emergence of eukaryotes 2 billion years ago brought the development of phosphorylation of serine/threonine and tyrosine residues as a means of signal transduction, although the development of dedicated protein tyrosine kinases is believed to have occurred with the emergence of multicellular eukaryotes to coordinate multicellular growth and development.

As organisms that possess all the characteristics of unicellular eukaryotes with regard to their modes of signal transduction, the impact of okadaic acid production in *P. lima* reversible protein phosphorylation remains unclear. Since okadaic acid seems to be sequestered away from cytoplasmic phosphatases, it is possible that the biosynthesis of this potent phosphatase inhibitor does not play a role in regulating signal transduction in this marine dinoflagellate. More detailed analysis of the biosynthesis and storage of okadaic acid in *P. lima* may provide insight into possible regulated interactions of the inhibitor with endogenous protein phosphatases. In the meantime, the technical obstacle of protein proteolysis in marine dinoflagellate cell extracts must be overcome before comprehensive biochemical analysis of this slow-growing toxic marine alga can be undertaken.

3.10 ■ BIBLIOGRAPHY

1. Hunter, T. (1987) A thousand and one protein kinases. *Cell* **50:** 823-829.

2. Hunter, T. (1995) Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80:** 225-236.

3. Hanks, S. K., Quinn, A.M., and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of catalytic domains. *Science* **241:** 42-52.

4. Cohen, P., and Cohen, P. T. W. (1989) Protein phosphatases come of age. *J. Biol. Chem.* **264:** 21435-21438.

5. Tonks, N. K. (1990) Protein phosphatases: key players in the regulation of cell function. *Curr. Opin. Cell Biol.* **2:** 1114-1124.

6. Barford, D. (1995) Protein phosphatases. *Curr. Opin. Struct. Biol.* **5:** 728-734.

7. Murakami, Y., Oshima, Y., and Yasumoto, T. (1982) Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. *Bull. Jpn. Soc. Sci. Fish.* **48:** 69-72.

8. Shimizu, Y. (1987) in The biology of dinoflagellates (Taylor, F. J. R., ed), pp. 282-316, Blackwell scientific publications, Oxford

9. Bialojan, C., and Takai, A. (1988) Inhibitory effects of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256:** 283-290.

10. Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K., Yamada, K., and Sugimura, T. (1988) Okadaic acid: An additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc. Natl. Acad. Sci. USA* **85:** 1768-1771.

11. Herschman, H. R., Lim, R. W., Brankow, D. W., and Fujiki, H. (1989) The tumor promoters 12-O-tetradecanoylphorbol-13-acetate and okadaic acid differ in toxicity, mitogenic activity and induction of gene expression. *Carcinogenesis* **10:** 1495-1494.

12. Murata, M., Shimatani, M., Sugitani, H., Oshima, Y., and Yasumoto, T. (1982) Isolation and elucidation of the causative toxin of the diarrhetic shellfish poisoning. *Bull. Jpn. Soc. Sci. Fish.* **48:** 549-552.

13. Rizzo, P. J. (1991) The enigma of the dinoflagellate chromosome. *J. Protozool.* **38:** 246-252.

14. Bujak, J. P., and Williams, G. L. (1981) The evolution of dinoflagellates. *Can. J. Bot.* **59:** 2077-2087.

15. Raikov, I. B. (1995) The dinoflagellate nucleus and chromosomes: Mesokaryote concept reconsidered. *Acta Protozool.* **34:** 239-247.

16. Cavalier-Smith, T. (1993) Kingdom protozoa and its 18 phyla. *Microbiol. Revs.* **57:** 953-994.

17. Boland, M. P., Taylor, M. F. J. R., and Holmes, C. F. B. (1993) Identification and characterisation of a type-1 protein phosphatase from the okadaic acid producing marine dinoflagellate *Prorocentrum lima*. *FEBS Lett.* **334:** 13-17.

18. Boland, M. P., and Holmes, C. F. B. (1994) Identification and characterisation of protein phosphatase-1 and -2A in marine dinoflagellates. *Adv. Prot. Phosphatases* **8:** 325-342.

19. Zhou, J., and Fritz, L. (1994) Okadaic acid antibody localizes to chloroplasts in the DSP-producing dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum*. *Phycologia* **33:** 455-461.

20. Hunter, T., and Cooper, J. A. (1985) Protein-tyrosine kinases. *Annu. Rev. Biochem.* **54:** 897-930.

21. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1990) Oncogenes and signal transduction. *Cell* **64:** 281-302.

22. Fischer, E. H., Charbonneau, H., and Tonks, N. K. (1991) The control of cellular signaling mechanisms by tyrosyl phosphoprotein phosphatases. *Science* **253:** 401-406.

23. Van Hoof, C., Goris, J., and Merlevede, J. (1993) Phosphotyrosine protein phosphatases: master key enzymes in signal transduction. *News Physiol. Sci.* **8:** 3-7.

24. Lundgren, K., Walworth, N., Boohu, R., Dembski, M., Kirschner, M., and Beach, D. (1991) *mik1* and *wee1* cooperate in the inhibitory tyrosine phosphorylation of *cdc2*. *Cell* **64:** 1111-1122.

25. Lim, M.-Y., Dailey, D., Martin, G. S., and Thorner, J. (1993) Yeast MCK1 protein kinase autophosphorylates at tyrosine and serine but phosphorylates exogenous substrates at serine and threonine. *J. Biol. Chem.* **269:** 21155-21164.

26. Lee, M. S., Enoch, T., and Piwnica-Worms, H. (1994) $mik1^{+}$ encodes a tyrosine kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. *J. Biol. Chem.* **261:** 30530-30537.

27. Featherstone, E. H., and Russel, D. (1991) Fission yeast $p107^{wee1}$ mitotic inhibitor is a tyrosine/serine kinase. *Nature* **349:** 808-811.

28. Gould, K. L., and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast cdc2 protein kinase regulates entry into mitosis. *Nature* **342:** 39-42.

29. Hide, G., Gray, A., Harrison, C. M., and Tait, A. (1989) Identification of an epidermal growth factor receptor homolog in trypanosomes. *Mol. Biol. Parasitol.* **36:** 51-60.

30. Wheeler-Alm, E., and Shapiro, S. Z. (1991) Evidence of tyrosine kinase activity in the protozoan parasite *Trypanosoma brucei*. *J. Protozool.* **39:** 413-416.

31. Torruella, M., Casano, L. M., and Vallejos, R. H. (1986) Evidence of the activity of tyrosine kinase(s) and of the presence of phosphotyrosine in pea plantlets. *J. Biol. Chem.* **261:** 6651-6653.

32. Cheng, H.-F., and Tao, M. (1989) Purification and characterization of a phosphotyrosyl-protein phosphatase from wheat seedlings. *Biochim. Biophys. Acta* **998:** 271-276.

33. Cool, D. E., and Blum, J. J. (1993) Protein tyrosine phosphatase activity in *Leishmania donovani*. *Mol. Cell. Biochem.* **127/128:** 143-149.

34. Guo, Y.-L., and Rous, S. J. (1995) Partial purification and characterization of an enzyme from pea nuclei with protein tyrosine phosphatase activity. *Plant Physiol.* **107:** 167-175.

35. Rodriguez, M., Cho, J. W., Sauer, H. W., and Rizzo, P. J. (1993) Evidence for the presence of a *cdc2*-like protein kinase in the dinoflagellate *Crypthecodinium cohnii*. *J. Euk. Microbiol.* **40:** 91-96.

36. Costas, E., and Lopez Rodas, V. (1991) On growth factors, cell division cycle and the eukaryotic origin. *Endocyt. Cell Res.* **8:** 89-92.

37. Mossman, K., Ostergaard, H., Upton, C., and McFadden, G. (1995) Myxoma virus and shope fibroma virus encode dual-specificity tyrosine/serine phosphatases which are essential for virus viability. *Virol.* **206:** 572-582.

38. Neufeld, E., Goren, H. J., and Boland, D. (1989) Thin-layer chromatography can resolve phosphotyrosine, phosphoserine, and phosphothreonine in a protein hydrolysate. *Anal. Biochem.* **177:** 138-143.

39. Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1988) Characterization of the major protein-tyrosine-phosphatase of human placenta. *J. Biol. Chem.* **263:** 6731-6737.

40. Brautigan, D. L., Bornstein, P., and Gallis, B. (1992) Phosphotyrosyl protein phosphatase: specific inhibition by Zn^{2+}. *J. Biol. Chem.* **256:** 6519-6522.

41. Van Belle, H. (1972) Kinetics and inhibition of alkaline phosphatases from canine tissues. *Biochim. Biophys. Acta* **289:** 159-168.

42. Van Etten, R. L. (1982) Human prostatic acid phosphatase: a histidine phosphatase. *Ann. N.Y. Acad. Sci.* **390:** 27-51.

43. Holmes, C. F. B., and Boland, M. P. (1993) Inhibitors of protein phosphatase-1 and -2A; two of the major serine/threonine protein phosphatases involved in cellular regulation. *Curr. Opin. Struct. Biol.* **3:** 934-943.

44. Murray, A., and Hunt, T. (1993) in The cell cycle: an introduction, pp. 42-55, Oxford University Press, New York

45. Hunter, T., and Plowman, G. D. (1997) The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.* **22:** 18-22.

46. Wilks, A. F. (1991) Cloning members of protein-tyrosine kinase family using polymerase chain reaction. *Methods Enzymol.* **200:** 533-546.

47. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253:** 407-414.

48. Taylor, S. S., and Adams, J. A. (1992) Protein kinases: coming of age. *Curr. Opin. Struct. Biol.* **2:** 743-748.

49. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S., and Zoller, M. J. (1993) A template for the protein kinase family. *Trends Biochem. Sci.* **18:** 84-89.

50. Taylor, S. S., Beuchler, J. A., and Yonemoto, W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* **59:** 971-1005.

51. Blumenthal, D. K., Takio, K., Hansen, R. S., and Krebs, E. G. (1986) Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent protein phosphatase. *J. Biol. Chem.* **261:** 8140-8145.

52. Døskeland, S. O., Maronde, E., and Gjertsen, B. T. (1993) The genetic subtypes of cAMP-dependent protein kinase - functionally different or redundant? *Biochim. Biophys. Acta* **1178:** 249-258.

53. Meinkoth, J. L., Alberts, A. S., Went, W., Fantozzi, D., Taylor, S. S., Hagiwara, M., Montminy, M., and Feramisco, J. R. (1993) Signal transduction through the cAMP-dependent protein kinase. *Mol. Cell. Biochem.* **127/128:** 179-186.

54. McKnight, G. S. (1991) Cyclic AMP second messenger systems. *Curr. Opin. Cell Biol.* **3:** 213-217.

55. Walsh, D. A., and Van Patten, S. M. (1994) Multiple pathway signal transduction by the cAMP-dependent protein kinase. *FASEB J.* **8:** 1227-1236.

56. Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990) The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. *Nature* **348:** 302-308.

57. Gonzalez, G. A., and Montminy, M. R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59:** 675-680.

58. Ma, H. (1993) Protein phosphorylation in plants: enzymes, substrates and regulators. *Trends Genet.* **9:** 228-230.

59. Carre, I. A., and Edmunds, L. N. (1992) cAMP-dependent kinases in the algal flagellate *Euglena gracilis*. *J.Biol. Chem.* **267:** 2135-2137.

60. Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987) Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* **50:** 277-287.

61. Mutzel, R., Lacombe, M.-L., Simon, M.-N., de Gunzburg, J., and Veron, M. (1987) Cloning and cDNA sequence of the regulatory subunit of cAMP-dependent protein kinase from *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* **84:** 6-10.

62. Hochstrasser, M., and Nelson, D. L. (1989) Cyclic AMP-dependent protein kinase in *Paramecium tetraurelia*. *J. Biol. Chem.* **264:** 14510-14518.

63. Mason, P. A., and Nelson, D. L. (1989) Cyclic AMP-dependent protein kinases of *Paramecium*. I. Chromatographic and physical properties of the enzymes from cilia. *Biochim. Biophys. Acta* **1010:** 108-115.

64. Dawson, J. F., Ostergaard, H. L., Klix, H., Boland, M. P., and Holmes, C. F. B. (1997) Evidence for phosphotyrosine signaling in the okadaic acid-producing marine dinoflagellate *Prorocentrum lima*. *J. Euk. Microbiol.* **44:** 89-95.

Chapter 4

Regulation of Protein Phosphatase-1 by cAMP-dependent Protein Kinase in Lower Eukaryotes*

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* Parts of this chapter have already been published:

Dawson, J. F., Wang, H. E., and Holmes, C. F. B. (1996) Identification and characterization of cAMP-dependent protein kinase and its possible direct interactions with protein phosphatase-1 in marine dinoflagellates. Biochem. Cell Biol. 74: 559-567.

Dawson, J. F., and Holmes, C. F. B. (1998) sds21 is present as an inhibitor-resistant high molecular mass complex and is not directly regulated by PKA. Biochem. Cell Biol., submitted.

4.1 ■ INTRODUCTION

Protein phosphatase type-1 (PP-1) is one of only four major protein serine/threonine phosphatases in eukaryotic cells responsible for regulating a plethora of physiological processes ranging from glycogen metabolism to gene expression to cell cycle control [1-4], as discussed in section 1.3. Understanding the regulation of PP-1 activity is therefore critical to a complete appreciation of signal transduction mechanisms governing fundamental processes in eukaryotic cells. Within the past decade, a growing family of natural product toxins, including the cyclic peptides microcystins and nodularins from marine and freshwater cyanobacterial sources [5] and the polyether fatty acid okadaic acid from marine dinoflagellates [6,7], have emerged as specific and potent inhibitors of PP-1 activity and may therefore serve as regulators of phosphatase activity.

My initial hypothesis was that investigating the nature of signal transduction in an organism that is a primary producer of one of these toxins, namely the okadaic acid-producing marine dinoflagellate *Prorocentrum lima*, may provide insight into how okadaic acid might regulate signal transduction in this organism and how the dinoflagellate is resistant to the effects of the toxin. During the course of my experiments, it was found that Prorocentrale dinoflagellates, including *P. lima*, *P. micans*, and *P. minimum*, possess PP-1 activity that is biochemically similar to their mammalian counterparts in their sensitivity to the marine toxins [8,9]. This evidence, taken together with the work described in the previous chapter, suggests that these marine dinoflagellates possess classical signal transduction mechanisms most similar to eukaryotes which are sensitive to the inhibitory effects of okadaic acid. Okadaic acid may also be compartmentalized within the dinoflagellate cytoplasm [10], and thus it appears that toxic dinoflagellates such as *P. lima* remain free from the effects of okadaic acid by sequestering it away from the enzymes that are most sensitive to it.

My examination of protein serine/threonine kinase activity in *P. lima* described in Chapter 3 demonstrated the presence of cAMP-dependent protein kinase (PKA) that was very similar in its biochemistry and structure to mammalian forms of the kinase. Analysis of dinoflagellate *P. micans* and *P. minimum* cell extracts in the presence of specific PKA agonists (cAMP) or inhibitors (PKI) demonstrated changes in the phosphorylation state of several proteins, suggesting the presence of PKA in these dinoflagellates as well. Intriguingly, the changes in phosphorylation state of these dinoflagellate proteins suggested that PKA regulates the activity of a protein phosphatase. Since the phosphorylation state of some of the same dinoflagellate phosphoproteins was also altered upon the addition of okadaic acid, which potently inhibits both PP-1 and PP-2A activity (see Table 1.2), I proposed that PKA might regulate PP-1 or PP-2A activity.

While the characterization of PKA activity in the Prorocentrale dinoflagellates was in progress, a 600 basepair fragment of the cDNA encoding PP-1 from *P. lima*, referred to herein as *P. lima* PP-1 (PLPP-1), was obtained. The sequence of this fragment revealed the presence of a classical PKA consensus sequence within PLPP-1. A sequence alignment of all known PP-1 sequences from diverse phylogenetic sources (Table 4.1) revealed that this PKA consensus sequence is prevalent among the lower eukaryotes. Furthermore, examination of the crystal structure of mammalian PP-1 that had just been released in the literature demonstrated that the homologous amino acid position in mammals (Asn-145 of PP-1α, see Table 1.3 and Fig. 1.10) is present at the surface of the molecule and is therefore accessible for possible molecular interactions.

Regulation of Protein Phosphatase Activity in Lower Eukaryotes by cAMP-dependent Protein Kinase

I therefore formulated the hypothesis that PP-1 from several lower unicellular eukaryotic organisms is phosphorylated by PKA and that this phosphorylation might regulate PP-1 activity. Regulation of some PP-1 isoforms by phosphorylation of the catalytic subunit is already known to occur both *in vitro* and *in vivo* through a C-terminal site that is acted upon by cdc2 kinase (see also section 1.5). In addition, we have already seen that PKA and PP-1 activities are intimately linked in several physiological processes among eukaryotes and that PP-1 activity commonly counteracts the effects of cAMP signaling through PKA. Alternatively, PKA regulates PP-1 activity in several instances by phosphorylating and activating inhibitor-1. As such, PKA regulates PP-1 indirectly through a polypeptide intermediate, in this case inhibitor-1. However, initial experiments failed to detect any inhibitor-1 activity in *P. lima* cell extracts, suggesting that if PKA regulates PP-1 activity in the dinoflagellate, it might do so directly by phosphorylating the catalytic subunit of PP-1 on the site found in the cDNA fragment of PLPP-1. I proposed that unicellular eukaryotes may possess simplified regulation of PP-1 by PKA through direct interactions. Metazoan eukaryotes may have evolved a more complex mechanism of PP-1 regulation by PKA through the development of inhibitor-1 (Fig. 4.1).

The work described in this chapter outlines experiments designed to test the hypothesis that PP-1 from lower eukaryotes is regulated directly by PKA through the phosphorylation of Ser-145. A peptide fragment of PLPP-1 surrounding the putative phosphorylation site was effectively phosphorylated by PKA and dephosphorylated by PP-2B, a scenario that parallels the regulation of inhibitor-1 activity in higher eukaryotes. This

Table 4.1 Alignment of known primary sequences of PP-1 from various organisms demonstrating the presence of a classical PKA consensus sequence (underlined with the phosphoacceptor site in bold) among lower eukaryotes. Amino acid residue numbers across the top are for rabbit PP-1α.

Higher Eukaryotes:	120	130	140	150	Reference
PP-1α Human	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[11]
PP-1α Rabbit	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[12]
PP-1α Rabbit-1	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[13]
PP-1α Mouse	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[14]
PP-1α Rat	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[15]
PP-1β Rabbit	LRGNHECASI	NRIYGFYDEC	KRRFNIKLWK	TFTD	[16]
PP-1β Human	LRGNHECASI	NRIYGFYDEC	KRRFNIKLWK	TFTD	[17]
PP-1β Mouse	LRGNHECASI	NRIYGFYDEC	KRRFNIKLWK	TFTD	[14]
PP-1β Rat	LRGNHECASI	NRIYGFYDEC	KRRFNIKLWK	TFTD	[18]
PP-1β Chicken	LRGNHECASI	NRIYGFYDEC	KRRFNIKLWK	TFTD	[19]
PP-1γ Human	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[20]
PP-1γ Mouse	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[14]
PP-1γ Rat	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[18]
PP-1γ *X. laevis*	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[21]
Higher Plants:					
A. thaliana 1	LRGNHESASI	NRIYGFYDEC	KRRFNVRLWK	IFTD	[22]
A. thaliana 2	LRGNHECASI	NRIYGFYDEC	KRRF**S**VRLWK	VFTD	[22]
A. thaliana 3	LRGNHECASI	NRIYGFYDEC	KRRY**S**VRVWK	IFTD	[22]
A. thaliana 4	LRGNHECASI	NRIYGFYDEC	KRRFNVRVWK	VFTD	[22]
A. thaliana 5	LRGNHECASI	NRIYGFYDEC	KRRFNVKLWK	VFTD	[22]
A. thaliana 6	LRGNHECASI	NRVYGFYDEC	KRRFNVKLWK	TFTD	[22]
Brassica oleracea	LRGNHECASI	NRIYGFYDEC	KRRFNVRLWK	IFTD	[23]
Zea mays	LRGNHECASV	NRIYGFYDEC	KRRF**S**VKLWK	TFTD	[24]
Medicago varia	LRGNHECASI	NRVYGFYDEC	KRRFNVKLWK	TFTD	[25]
Oryza sativa	LRGNHECASI	NRIYGFYDEC	KRRFNMRVWK	VFTD	[26]
Phaseolus vulgaris	LRGNHECASI	NRVYGFYDEC	KRRYNVKLWK	IFTD	[27]
Insects:					
D. melanogaster α1	LRGNHECASI	NRIYGFYDEC	KRRY**T**IKLWK	TFTD	[16]
D. melanogaster α2	LRGNHECASI	NRIYGFYDEC	KRRY**S**IKLWK	TFTD	[28]
D. melanogaster α3	LRGNHECASI	NRIYGFYDEC	KRRY**T**IKLWK	TFTD	[16]
D. melanogaster β	LRGNHECASI	NRIYGFYDEC	KRRYNVKLWK	TFTD	[16]
Lower Eukaryotes:					
S. cerevisiae	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[14]
S. pombe dis2	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[14]
S. pombe sds21	LRGNHEFASI	NRIYGFYDEC	KRRY**S**IKLWK	TFTD	[14]
A. cliftonii-1	LRGNHECASI	NRIYGFYDEC	KRRYNIRLWK	TFTD	[29]
A. cliftonii-2	LRGNHECASI	NRIYGFYDVC	KRRYNIRLWK	TFTD	[29]
A. nidulans	LRGNHECASI	NRIYGFYDEC	KRRYNIKLWK	TFTD	[30]
T. brucei-1	LRGNHECSSI	NRIYGFFDEC	KRRY**S**VRLWK	QFTD	[31]
T. brucei-2	LRGNHECSSI	NRIYGFFDEC	KRRY**S**VRLWK	QFTD	[31]
Paramecium	LRGNHECASI	NRIYGFYDEC	KRRY**T**IKLWK	TFTD	[32]
Prorocentrum lima	LRGNHECASI	NRIYGFYDEC	KRRY**S**IRLWK	TFTD	

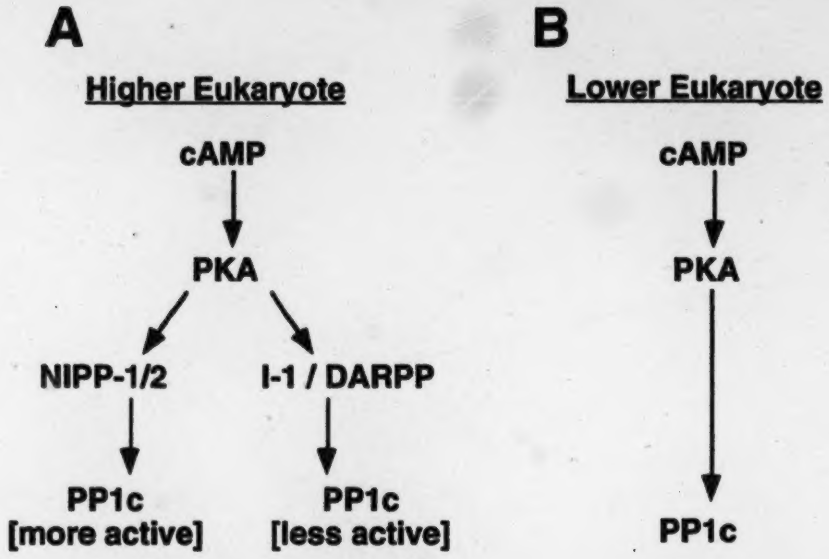


Fig. 4.1 Hypothetical development of protein phosphatase-1 regulation by cAMP-dependent protein kinase. **A.** In higher eukaryotes, PKA regulates PP-1c indirectly via phosphorylation of the inhibitor proteins I-1 and its neuronal homolog DARPP-32 and the nuclear proteins NIPP-1/2. **B.** In lower eukaryotes such as *Prorocentrum lima*, where evidence for I-1 is lacking, PKA may regulate PP-1c directly via phosphorylation of the catalytic subunit of the phosphatase.

encouraging result lead to the decision to examine my hypothesis in the fission yeast *Schizosaccharomyces pombe*, which possesses two isoforms of PP-1. One isoform, termed sds21, possesses the putative PKA phosphorylation site, while its counterpart, referred to as dis2, does not. dis2 has already been shown to be phosphorylated and regulated by cdc2 kinase and therefore the *S. pombe* system has proven to be amenable to studies aimed at examining the potential for PP-1 regulation by protein phosphorylation. In addition, the *S. pombe* system is readily exploited by documented biochemical and molecular biological techniques and is therefore an attractive alternative to the relatively unknown system of the marine dinoflagellates. For example, mutants of *S. pombe* in which either the sds21 or dis2 gene was knocked-out had already been constructed and were available to study [33].

4.2 ■ MATERIALS AND METHODS

Strains, Media, and Chromatography

The YEG17 *S. pombe* strain (a gift from Dr. C. Rasmussen, University of Saskatchewan, Canada) was used as wild type. The cDNA for the *sds21* isoform of protein phosphatase-1 from *S. pombe* and Δdis2 (*h*$^-$ *leu1-32 ura4-D18 dis2::ura4*$^+$) and Δsds21 (*h*$^-$ *leu1-32 ura4-D18 sds21::ura4*$^+$) *S. pombe* were generous gifts from Dr. M. Yanagida, Kyoto University, Japan. Monoclonal antibody 12CA5 was obtained from Boehringer-Mannheim (Montreal, Canada). Oligonucleotides were synthesized in the DNA Core Facility, Department of Biochemistry, University of Alberta. DEAE-Sepharose, Heparin-Sepharose, Mono-Q, Source-Q 15, and Superdex-75 were purchased from Pharmacia (Uppsala, Sweden).

Preparation of Peptides

Kemptide was purchased from Sigma. R_{II} peptide was a gift from Drs. T. Batuik and P. Halloran, University of Alberta. PLPP-1 peptide was synthesized by the Alberta Peptide Institute, Edmonton, Alberta. PPIP46 and PP-1G peptides were prepared as outlined in Chapter 2. All peptide solutions were analyzed by amino acid analysis to determine their exact concentration. Radiolabeling of peptides for calcineurin assays was carried out as outlined in [34].

PKA and Calcineurin Assays

Peptide phosphorylation assays were performed as follows: 1 unit PKA (catalytic subunit from bovine heart, Sigma) was added to a reaction mixture consisting of 50 mM Tris-HCl pH 7.5, 0.4 mg/mL BSA, 0.3 mM DTT, 20 μM peptide substrate, 1 mM $MgCl_2$, and 1.67 μM [γ-^{32}P] ATP (specific activity of 3000 Ci/mmol) in a total volume of 200 μL and incubated at 30 °C. Aliquots (18 μL) were removed at 1 minute intervals for 10 minutes and dotted onto P-81 phosphocellulose strips (Whatman). The phosphocellulose strips were then washed in 0.75 M H_3PO_4 and placed in capless Eppendorf tubes into which scintillation fluid (Aqueous Counter Scintillant, Amersham) was added prior to counting in a scintillation counter (1209 rackbeta, Wallac).

Peptide dephosphorylation assays were carried out as follows: 2 units of calcineurin (PP-2B, Sigma) were added to a reaction mixture consisting of 1 mM $CaCl_2$, 1 μM calmodulin, 0.4 mg/mL BSA, 0.3 mM DTT, and 12.5 x 10^4 cpm phosphopeptide substrate in a total volume of 75 μL and incubated at 30 °C. Aliquots (12 μL) were removed at 1 minute intervals for 5 minutes and dotted onto P-81 phosphocellulose strips (Whatman). The phosphocellulose strips were then treated as above and counted on a scintillation counter.

Preparation of Cell Extracts

S. pombe cells were collected and 2.5 mL Solution A (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 1.0 mM PMSF, 1.0 mM benzamidine, 0.1% β-mercaptoethanol (v/v), and 10% glycerol (v/v)) containing a cocktail of protease inhibitors described in [8] including 0.5 μg/mL soybean trypsin inhibitor and 0.75 μg/mL TPCK was added per gram wet weight cell pellet. Cell suspensions were then passed through a French press twice at 16000 psi. Resulting homogenates were centrifuged at 16000 *g* (r_{av}=7.3 cm) for 20 min at 4 °C and used immediately.

Phosphorylase Phosphatase Assay

Phosphorylase phosphatase activity was determined as described in [35]. Samples to be tested were diluted to 20 μL with Solution B (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mg/mL BSA and 0.2% β-mercaptoethanol (v/v)). ^{32}P-labeled phosphorylase *a* was routinely prepared by phosphorylating phosphorylase *b* (Sigma) with phosphorylase kinase (Sigma) as outlined in [36]. Phosphorylase *a* (50 mg/mL) was diluted to 3 mg/mL (30 pmol/μL) in Solution B containing 15 mM caffeine. An aliquot (10 μL) of this mixture containing ^{32}P-labeled phosphorylase *a* (specific radioactivity > 0.6 x 10^6 dpm/nmol) was added and the reaction allowed to proceed for 10 min at 30 °C. The reaction was terminated by the addition of 200 μL of trichloroacetic acid (20% w/v) to precipitate phosphorylase *a* and the suspension mixed with a vortex mixer followed by incubation on ice for 1 min prior to centrifugation at 16000 *g* (r_{av}=7.3 cm) for 2 min. An aliquot (200 μL) of the supernatant was added to 1 mL scintillant and counted. Protein phosphatase activity is then expressed as a function of the percentage of ^{32}P released from the labeled phosphorylase *a*. Under specific conditions, one milli unit (mU) of protein phosphatase activity releases 1 nmole phosphate from phosphorylase *a* per min.

The kinetics involved with this assay require some comment. The K_M of both PP-1 and PP-2A for phosphorylase *a* is approximately 5 μM [36]. The concentration of phosphorylase in each assay is approximately 10 μM, which is twice the K_M of the phosphatase. Under these conditions, there is no lag phase upon addition of substrate and the assay is linear with time up to 30% ^{32}P released from the labeled phosphorylase. Therefore, to accurately calculate an amount of phosphatase activity, dilutions of a sample containing an unknown amount of phosphatase must be diluted until less than 30% ^{32}P release is achieved and the amount of phosphatase activity back-calculated to the original sample concentration. As a general rule, an amount of phosphatase activity causing 20% release of ^{32}P label was employed when producing dose response curves for the marine toxins. While screening chromatography fractions for the presence of phosphorylase phosphatase activity, enzyme activity was reported as the percentage ^{32}P released to identify those fractions that contain activity. In addition, since chromatographic fractions contain complex mixtures of different proteins, accurate quantitative assessment of the amount of enzyme in these fractions is often difficult.

Toxin Dose Response Curves

In this Chapter, I introduce the concept of employing marine toxins to determine the identity of unknown protein serine/threonine phosphatase activity. The substrate phosphorylase is an excellent substrate for both PP-1 and PP-2A, as noted above. Therefore, in a complex mixture such as cell lysates, one is unable to distinguish between PP-1 and PP-2A activity when phosphorylase is employed as substrate. To aid in the identification of activities separated by FPLC, I produced dose response curves for fractions that contained phosphorylase phosphatase activity. As noted in Table 1.2, PP-2A has the same IC_{50} (concentration of inhibitor which elicits 50% inhibition of an enzyme relative to control enzyme activity in the absence of inhibitor under standardized conditions) for both microcystin-LR and okadaic acid. Therefore, dose response curves for PP-2A employing the two marine toxins should overlap. Conversely, the IC_{50} of okadaic acid is 100-fold greater than that of microcystin-LR when PP-1 is inhibited. Dose response curves for PP-1 should reflect this difference and the curves should be offset by a factor of approximately 100. IC_{50} values can be determined by several means including: 1) inspection of dose response curves, 2) calculation based on points of dose response curves in the linear range of the curve, and 3) direct measurement by dilution of inhibitor until 50% inhibition is acheived.

The kinetics of inhibition of PP-1 and PP-2A by the marine toxins also require some comment. The marine toxins microcystin-LR, nodularin, and okadaic acid all inhibit the phosphatases with IC_{50} values in the nanomolar range. These values reflect the high affinity with which phosphatases bind the marine toxins. The marine toxins are classified as "tight-binding inhibitors" [37]. These marine toxins bind their phosphatase targets such that the dose response is very sensitive to the concentration of enzyme in an assay, rather than the concentrations of inhibitor and substrate, which is the classical case described by Michaelis-Menton kinetics [38]. The tight-binding marine toxins are assumed to bind to the target enzyme immediately resulting in a stable inactive complex. The toxins are also assumed to form complexes with PP-1 with 1:1 stoichiometry. Therefore, the stoichiometry of inhibitor to enzyme in an assay is a reflection of the amount of inhibition of enzyme activity. For example, at the IC_{50} for a given tight-binding toxin, the stoichiometry of toxin to enzyme in the assay is ideally 1:2. Therefore, with tight-binding inhibitors, there is always more enzyme than inhibitor over the range of detectable activity. Once the concentration of inhibitor and enzyme are equal, there should theoretically be no measureable activity.

To obtain accurate IC_{50} information, precise enzyme quantitation is required. For complex mixtures such as chromatographic fractions of cell lysates, this is very difficult. Therefore, dose response curves determined for chromatographic fractions containing phosphorylase phosphatase activity are informative as long as the same amount of activity is included in each assay. The precise IC_{50} value that is arrived at is not as important as how the dose response curves for microcystin-LR and okadaic acid relate to one another. However, as we shall see, even with complex mixtures, the approximate IC_{50} value that is obtained is very close to that of pure enzyme.

Often, when comparing the dose response curves of purified protein, it is more convenient to employ a standard amount of phosphatase activity in the form of a standard percentage of ^{32}P released, so long as the specific activity of the phosphatases employed is similar. This is the case with the dose response curves generated in Chapter 5.

Fast Performance Liquid Chromatography

S. pombe cell extracts were applied to a 2.0 cm x 8 cm DEAE-Sepharose column equilibrated in Solution A, developed with a linear gradient from 0 to 0.5 M NaCl in Solution A and 20 µL of each fraction (5 mL) was assayed for phosphorylase *a* phosphatase activity as outlined above. Dose response curves for fractions containing phosphorylase phosphatase activity were determined by assaying in the presence of 0.3 to 1000 nM microcystin-LR or okadaic acid (see previous section). PP-1-containing fractions were pooled, rechromatographed on a Mono-Q strong anion exchange column (1.0 cm x 5.0 cm, Pharmacia) developed with a linear gradient from 0 to 0.5 M NaCl in Solution A, and 20 µL of each fraction (1 mL) assayed as described above. Active Mono-Q fractions were then pooled and rechromatographed on either a Superdex-200 or Superdex-75 gel filtration column (1.0 cm x 30 cm, Pharmacia) with 0.2 M NaCl in Solution A and 20 µL of each fraction (200 µL) assayed as above.

Construction of Vectors and Mutants

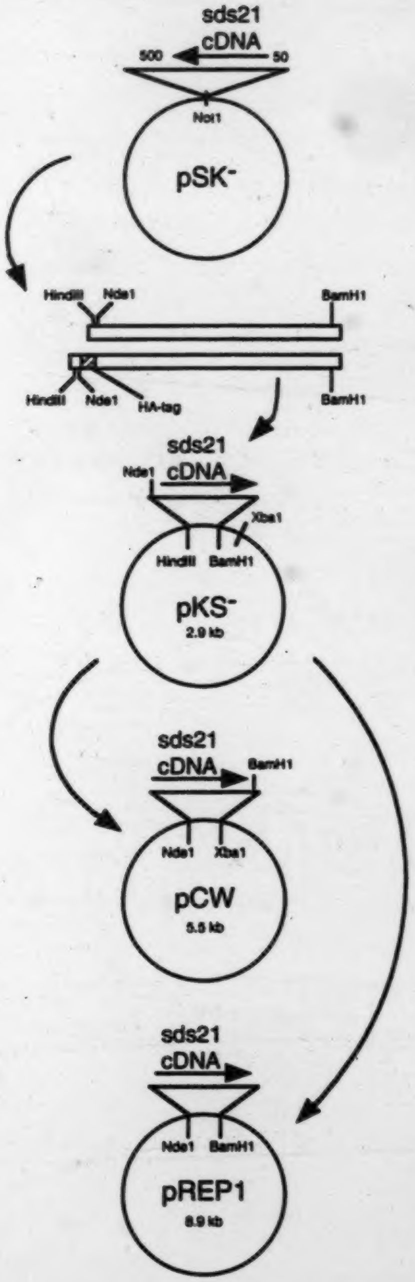
The general strategy for the construction of vectors employed is outlined in Fig. 4.2. The coding sequence for *sds21* was isolated by the polymerase chain reaction (PCR) amplification using the sense 5' primer 5'-CGCGCAAGCTTCATATGGATTATGATATTGATGCG-3' (HindIII and NdeI restriction sites underlined) and antisense 3' primer 5'-GCGCGGATCCTAATTATTTTTGGATTTCTTC-3' (BamH1 restriction site underlined). The oligonucleotides employed in this work are illustrated in Fig. 4.3. Oligonucleotides encoding the HA-epitope sequence YPYDVPDYA were inserted in frame with *sds21* by PCR amplification employing the sense 5' primer 5'-CGCGCAAGCTTCATATG*TATCCATATGATGTTCCAGATTATGCT*GATTATGATATTGATGCG-3' (HindIII and NdeI restriction sites underlined, HA-epitope coding sequence in italics) and the antisense 3' primer described above. The PCR fragments were subcloned into the pKS$^-$ vector (Stratagene) using the HindIII and BamH1 restriction sites to produce either pKS-sds21 or pKS-HAsds21.

The S145A substitution mutant was constructed using the Quikchange Mutagenesis System (Stratagene) with the following oligonucleotide and its antisense oligonucleotide: 5'-GCGTCGGTAT*GC*CATAAGGCT*T*TGGAAAACCTTTACCC-3' (mismatches in italics, HindIII restriction site underlined). DNA sequencing was performed to verify the correctness of all the constructs produced. The *sds21* coding sequence contained in the NdeI-XbaI fragment from wild type or mutant pKS$^-$-*sds21* or pKS-HAsds21 was then subcloned into the pCW expression vector for expression in *E. coli*. Alternatively, the NdeI-BamH1 fragment of wild type or mutant pKS-sds21 or pKS-HAsds21 was subcloned into the *S. pombe* pREP-1 expression vector (a gift from Dr. C. Rasmussen, University of Saskatchewan).

Expression and Purification of sds21 from *E. coli*

Expression of sds21 or epitope-tagged sds21 was performed in *E. coli* DH-5α host cells (Life Technologies) grown at 37 °C in 100 mL Luria Broth (LB media) containing 1 μM MnCl$_2$, 0.1% vitamin B1 (w/v) and 200 μg/mL ampicillin. Cells were grown at 37 °C until an optical density at 600 nm of 0.5 to 0.6 was reached. Expression was then induced by the addition of 0.5 mM isopropyl β-thiogalactoside (IPTG) followed by growth at 37 °C overnight.

sds21 was then purified from inclusion bodies following a protocol adapted from Berndt and Cohen [39]. Cell lysates were produced by suspension of collected cells in 4 mL Buffer A (50 mM imidazole, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 2.0 mM MnCl$_2$, 0.5 mM PMSF, 2.0 mM benzamidine, 0.1% β-mercaptoethanol (v/v), and 10% glycerol


sds21
cDNA
500
50
Not1
pSK-
HindIII
Nde1
BamH1
HindIII
Nde1
HA-tag
BamH1
sds21
cDNA
Nde1
Xba1
HindIII
BamH1
pKS-
2.9 kb
sds21
cDNA
BamH1
Nde1
Xba1
pCW
5.5 kb
sds21
cDNA
Nde1
BamH1
pREP1
8.9 kb

pKAO-3 starting plasmid: 50 bp and ca. 500 bp flanking sequences up- and downstream of sds21 cDNA, respectively, in pSK⁻ vector.

PCR cDNA inserting HindIII and Nde1 sites at 5' end and BamH1 at 3' end. Make 2 5' end primers: one containing the HA-tag.

Ligate PCR products into pKS⁻ restricted at HindII and BamH1.
Sequence cDNA to confirm correct sequence.

Ligate Nde1-Xba1 fragment into pCW vector for expression in *E. coli.*

Ligate Nde1-BamH1 fragment into pREP1 vector for expression in *S. pombe.*

Fig. 4.2 Construction of vectors for expression of sds21 and hemagluttinin epitope (HA)-tagged sds21 in *E. coli* using the pCW expression vector and *S. pombe* using the pREP-1 expression vector. See the Materials and Methods section for a complete description.

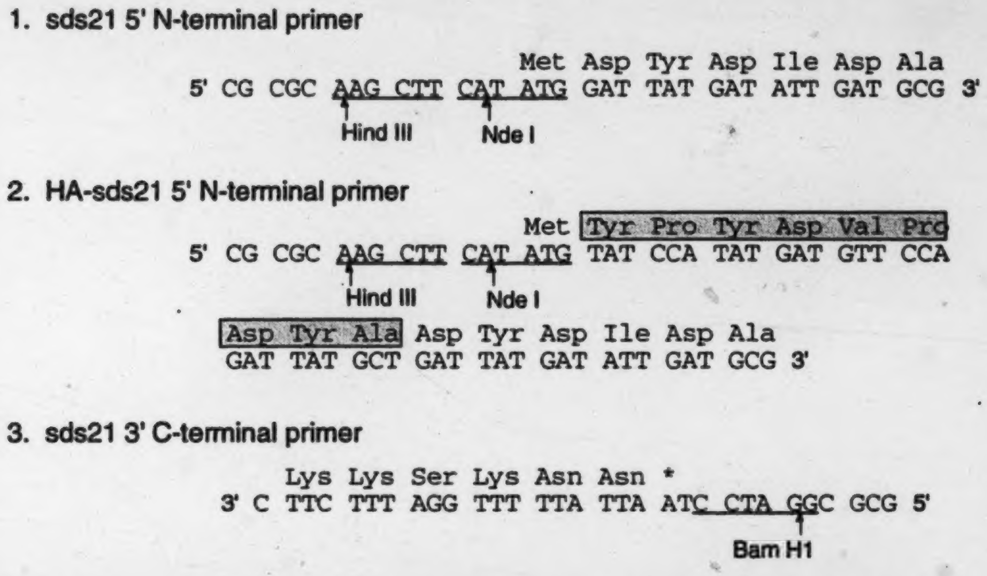

1. sds21 5' N-terminal primer
Met Asp Tyr Asp Ile Asp Ala
5' CG CGC AAG CTT CAT ATG GAT TAT GAT ATT GAT GCG 3'
Hind III
Nde I
2. HA-sds21 5' N-terminal primer
Met Tyr Pro Tyr Asp Val Pro
5' CG CGC AAG CTT CAT ATG TAT CCA TAT GAT GTT CCA
Hind III
Nde I
Asp Tyr Ala Asp Tyr Asp Ile Asp Ala
GAT TAT GCT GAT TAT GAT ATT GAT GCG 3'
3. sds21 3' C-terminal primer
Lys Lys Ser Lys Asn Asn *
3' C TTC TTT AGG TTT TTA TTA ATC CTA GGC GCG 5'
Bam H1

Fig. 4.3 Oligonucleotide primers employed to amplify sds21 and HA-tagged sds21 coding sequences utilizing PCR. Restriction sites are underlined and labelled. The HA-tag epitope sequence is shaded. See the Materials and Methods section for a full description.

(v/v)) containing 100 mM NaCl followed by disruption in a French press at 16000 psi. Pellets resulting from centrifugation of cell lysates were washed in a Triton X-100-containing buffer and solubilized in a buffer containing 6 M guanidinium chloride. sds21 was renatured by quickly adding a 50-fold volume of renaturation buffer containing 0.8 M NaCl and 1 mM $MnCl_2$ to the solubilized protein as outlined in Berndt and Cohen [39].

The renaturation reaction was then dialyzed against one change of an eight-fold volume of renaturation buffer containing no salt in a cold room (ca. 8 °C) overnight. Resulting dialysates were centrifuged at 16000 *g* (r_{av}=7.3 cm) for 20 min at 4 °C and the supernatants applied onto a 5 mL HiTrap Heparin-Sepharose column (Pharmacia). Following equilibration with Buffer A containing 100 mM NaCl, sds21 was eluted in Buffer A by step-wise increases in salt concentration from 200 mM to 450 mM to 600 mM. sds21 activity eluted in the lead fractions containing 450 mM NaCl. Active fractions (5 mL) were pooled and concentrated in a centriprep-10 concentrator (Amicon) prior to dialysis against storage buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 1.0 mM $MnCl_2$, 50% glycerol (v/v), 6 mM DTT, and 1.0% β-mercaptoethanol (v/v)) and storage at -20 °C.

Expression and Purification of sds21 and HA-sds21 from *S. pombe*

pREP-1-sds21 or pREP-1-HAsds21 was transformed into Δdis2 (*h⁻ leu1-32 ura4-D18 dis2::ura4⁺*) *S. pombe* by the alkali cation method [40]. Transformed *S. pombe* were grown in 100 mL of MBS media (MB media containing 150 μg/mL adenine and uracil) with 0.1 mM thiamine at 30 °C to a cell density of 3 x 10^6 cells/mL. Cells were then collected and washed in sterile water followed by two washes in MBS media, resuspended in 100 mL MBS media and grown with shaking at 30 °C overnight. 5.0 x 10^9 cells were then collected and resuspended in 4 mL Buffer A containing 100 mM NaCl, a cocktail of protease inhibitors outlined in [8] including soybean trypsin inhibitor (0.5 μg/mL) and TPCK (0.75 μg/mL) prior to cell disruption in a French press at 16 000 psi. Cell lysates were then cleared by centrifugation at 16000 *g* (r_{av}=7.3 cm) for 10 min at 4 °C. Resulting supernatants applied to a 5 mL HiTrap Heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with Buffer A containing 100 mM NaCl were developed with a linear gradient from 100 to 600 mM NaCl Buffer A at a linear flow rate of 5 mL/min.

Heparin-Sepharose fractions (5 mL) were then assayed for the ability to dephosphorylate ^{32}P-radiolabelled glycogen phosphorylase *a* as described above. Phosphorylase phosphatase-containing fractions from Heparin-Sepharose chromatography were pooled, diluted in a three-fold volume of Buffer B (50 mM imidazole, pH 7.2, 0.5 mM EDTA, 0.5 mM EGTA, 3.0 mM $MnCl_2$, 0.5 mM PMSF, 2.0 mM benzamidine, 0.1% β-mercaptoethanol (v/v), and 10% glycerol (v/v)), and applied to a Source-Q 15 column (1.0 cm x 2.0 cm) equilibrated in Buffer B. The column was then developed with a linear gradient of 0 to 400 mM NaCl in Buffer B at a linear flow rate of 1 mL/min. Aliquots (5 μL) of source-Q fractions (1 mL) were then assayed for phosphorylase phosphatase activity as outlined above.

Phosphorylase phosphatase-containing fractions from Source-Q 15 chromatography were concentrated in a centriprep-10 (Amicon) concentrator to a volume of 0.5 mL and applied to a Superdex-75 gel filtration column (Pharmacia) equilibrated in Buffer A containing 200 mM NaCl and eluted in the same buffer at a linear flow rate of 0.5 mL/min. Aliquots (2 μL) of Superdex-75 fractions (200 μL) were assayed for phosphorylase phosphatase activity as described above. Phosphorylase phosphatase-containing gel filtration fractions were then pooled and dialyzed against storage buffer and stored at -20 °C. The final yield of sds21 or HA-sds21 purified from *S. pombe* in this manner was 13 μg from 5.0 x 10^9 cells. Protein determinations were performed by the Bradford method [41].

Western Immunoblotting

Induced and uninduced *E. coli* cultures (1 mL) harbouring the pCW-sds21 or pCW-HAsds21 plasmid and grown to 6 h post-induction at 37 °C were harvested by centrifugation and 50 µL Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol (v/v), 20% SDS (w/v), 5% β-mercaptoethanol (v/v), and 0.00625% bromophenol blue (w/v)) added. Following incubation at 85 °C for 10 min, the mixture was spun briefly in a benchtop microcentrifuge and 10 µL run on SDS-PAGE (12%) alongside 0.13 µg of sds21 and HA-sds21 expressed and purified from *S. pombe* as described above. Proteins were transferred to Hybond-C extra nitrocellulose (Amersham). Nitrocellulose was incubated with anti-HA antibody (12CA5, mouse monoclonal antibody, Boehringer Mannheim) and bound mouse IgG was detected using horseradish peroxidase-conjugated rabbit-anti mouse IgG (Amersham) and visualized employing the ECL Western blotting kit (Amersham).

cAMP-dependent Protein Kinase assays

PKA (10 units, catalytic subunit from bovine heart, Sigma) was added to a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 12.5 mM DTT, 0.8 µM [γ-^{32}P] ATP (specific activity of 3000 Ci/mmol, Amersham), and either sds21 or S145A sds21 mutant (1.0 µg). The reaction was stopped by the addition of Laemmli buffer followed by incubation at 85 °C for 10 min. Samples were then loaded and run on SDS-PAGE (12%), dried on a Biorad Gel Drier, and exposed to X-ray film (Kodak) to produce an autoradiogram. Prestained molecular mass standards (Biorad) were used.

4.3 ■ RESULTS

A Peptide Fragment of PLPP-1 is Phosphorylated by cAMP-dependent Protein Kinase and Dephosphorylated by Calcineurin

A fragment of the *P. lima* PP-1 primary sequence (PLPP-1 peptide) surrounding the presumptive PKA site was synthesized to determine if it could be phosphorylated by PKA and dephosphorylated by calcineurin (PP-2B). PLPP-1 peptide phosphorylation and dephosphorylation was compared to four other known peptide substrates: Kemptide, R_{II} peptide, PPIP-46 peptide, and PP-1G peptide (Table 4.2). These peptides represent physiological substrates of PKA [42-44]. Kemptide corresponds to a fragment of pyruvate kinase, R_{II} peptide is from the R_{II} regulatory subunit of PKA, PPIP-46 is the active fragment of inhibitor-1, and PP-1G corresponds to a fragment of the glycogen binding subunit of PP-1. The PLPP-1 peptide was phosphorylated by PKA at a rate comparable to that of the other three known peptide substrates (Fig 4.4A). This is consistent with the possibility that *P. lima* PP-1 may be a substrate for PKA *in vivo*.

Table 4.2 Sequences of PKA substrates analyzed for their ability to be phosphorylated by PKA and dephosphorylated by calcineurin (PP-2B) (see Fig. 4.4). The PKA consensus sequence (see Table 1.1) is underlined while phosphoacceptor site is indicated in bold.

Name	Sequence
Kemptide	LRRA**S**LG
R_{II} Peptide	DLDVPIPGRFDRRV**S**VAAE
PPIP-46	KIQFTVPLLEPHLDPEAAEQIRRRRP**T**PATLVLTSDQSSPEVDEDR
PP-1G	SPSQPSRRG**S**ESSEE
PLPP-1	NRIYGFYDECKRRY**S**IRLWKTSTD

Both Kemptide and PP-1G phosphopeptides were not dephosphorylated by calcineurin and are therefore not substrates for this protein phosphatase (Fig. 4.4B). Conversely, R_{II} and PPIP-46 phosphopeptides were efficiently dephosphorylated by calcineurin, as expected [42-45]. PLPP-1 phosphopeptide was also dephosphorylated by calcineurin, although not as efficiently as R_{II} or PPIP-46. These results are consistent with the concept that *P. lima* PP-1 may be a substrate for calcineurin *in vivo*.

The phosphorylation of the PLPP-1 peptide by PKA was also analyzed employing the capillary electrophoresis-based assay outlined in Chapter 2 (Fig. 4.5). The results of these assays verified that the PLPP-1 peptide is efficiently phosphorylated by PKA. These results employing the PLPP-1 peptide were very encouraging as they indicated that the putative site of phosphorylation on PP-1 may be targeted by PKA *in vivo*. I therefore undertook to examine the hypothesis that PKA may phosphorylate and regulate PP-1 activity among several lower eukaryotes. I decided to investigate PP-1 in the fission yeast *Schizosaccharomyces pombe* since this organism contains two isoforms of PP-1, one (termed sds21) possessing the putative phosphorylation site and another (referred to as dis2) not. In addition, the extensive biochemical and molecular biological techniques that have already been developed in this system were an attractive alternative to working with the relatively unknown system of marine dinoflagellates, as discussed in section 4.1.

Characterization of Protein Phosphatase-1 in *S. pombe*

My initial efforts were aimed at purifying native sds21 enzyme from *S. pombe* to use in phosphorylation experiments. I also set out to purify native dis2 from *S. pombe* as a control PP-1 enzyme that does not possess the putative PKA phosphorylation site. To

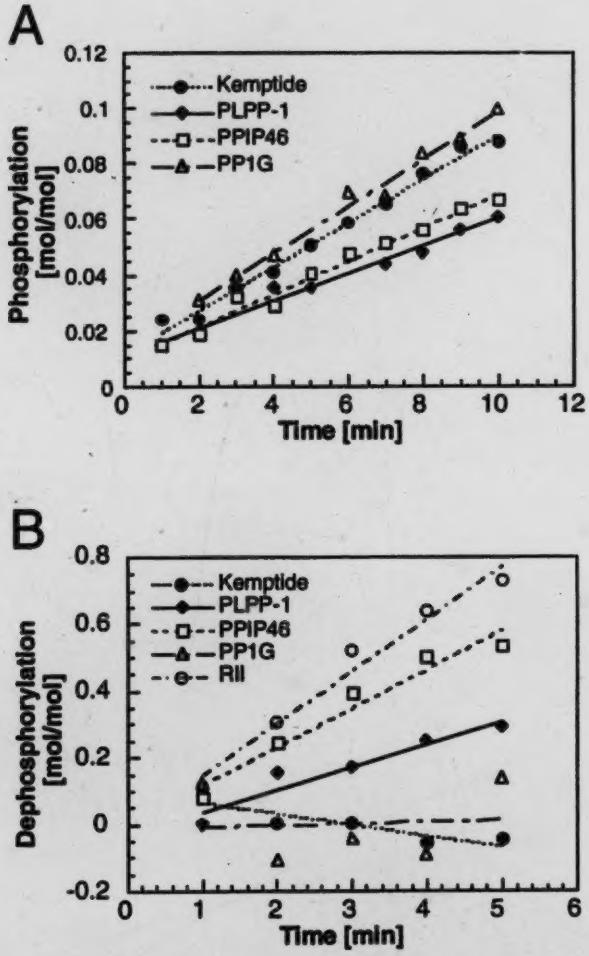


Fig. 4.4 PLPP-1 peptide is phosphorylated by PKA and dephosphorylated by calcineurin (PP-2B). **A.** Peptide substrate phosphorylation by PKA. **B.** Peptide substrate dephosphorylation by calcineurin (PP-2B). The primary sequences of the peptide substrates employed are listed in Table 4.2. Substrate (20 μM) and 0.1 units PKA or 2 units calcineurin were included in each assay, as outlined in the Materials and Methods section.

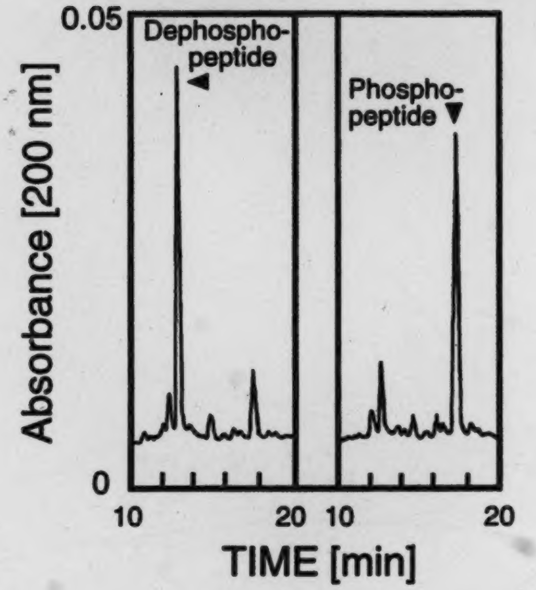


Fig. 4.5 Detection of PLPP-1 peptide (see Table 4.2) phosphorylation by cAMP-dependent protein kinase employing the capillary electrophoresis-based assay described in Chapter 2. PKA (25 units) was employed in the phosphorylation reaction. Dephosphopeptide is seen at time 0.1 min of the reaction in the left panel, while a significant peak of phosphorylated PLPP-1 peptide is detected at time 0.5 min under these conditions. The total amount of PLPP-1 peptide applied to the capillary was 0.2 pmol.

facilitate this work, I obtained the dis2 and sds21 knock-out strains of *S. pombe* from Dr. M. Yanagida at Kyoto University in Japan.

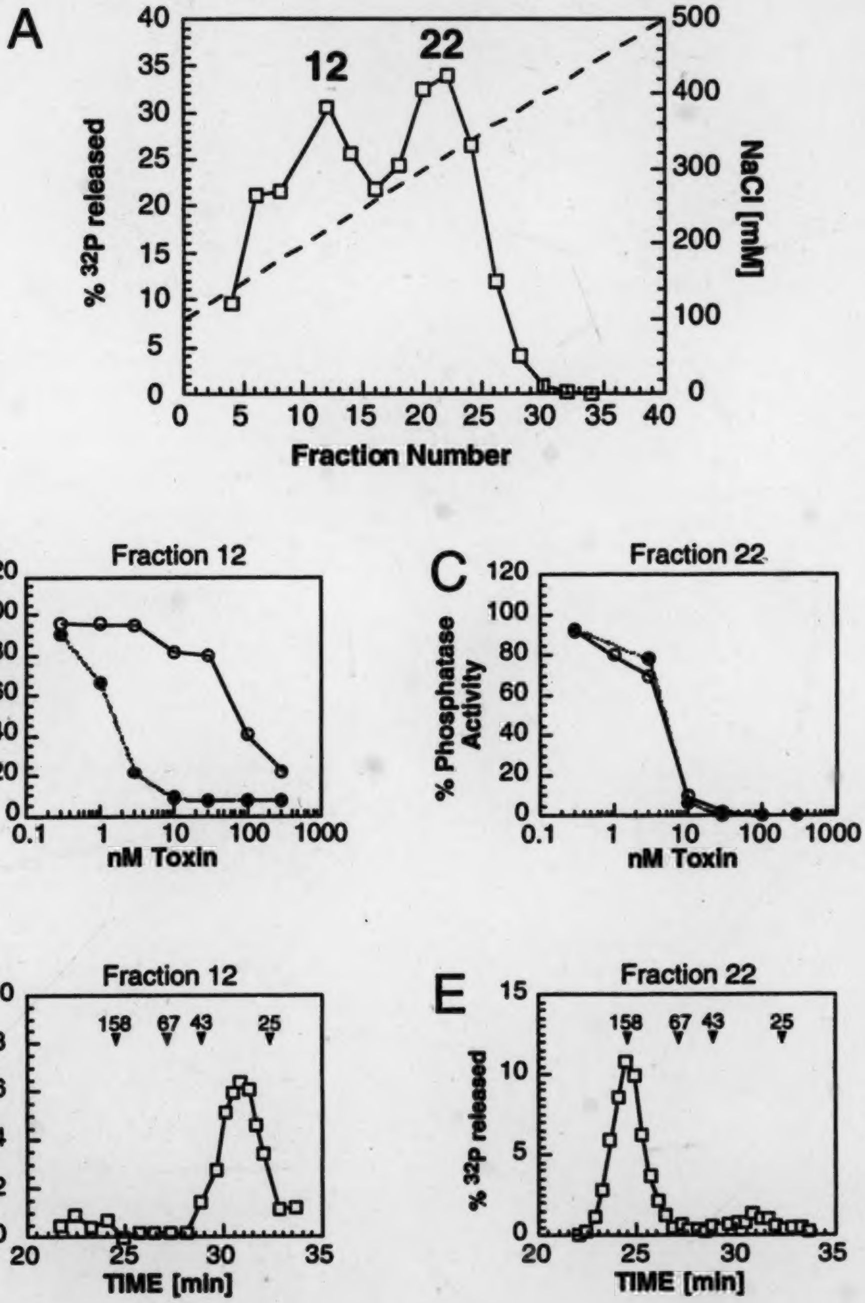
Preliminary purification trials employing cell extracts of the YEG17 strain of *S. pombe* possessing both sds21 and dis2 genes revealed the presence of two peaks of phosphorylase phosphatase activity off DEAE-Sepharose weak anion exchange chromatography (Fig. 4.6A). Since phosphorylase is a specific substrate for both PP-1 and PP-2A, the activities within the DEAE fractions were subjected to dose inhibition characterization employing microcystin-LR and okadaic acid. As discussed in section 1.6, microcystin-LR is an equipotent inhibitor of both PP-1 and PP-2A with an IC_{50} value of 0.2 nM against both phosphatases (see also Table 1.2). Similarly, the IC_{50} of okadaic acid against PP-2A is the same as that for microcystin-LR, being 0.2 nM. Conversely, okadaic acid inhibits PP-1 with an IC_{50} 100-fold greater than that of microcystin-LR, being 20 nM. Therefore, the IC_{50} curves for PP-2A employing microcystin-LR and okadaic acid should overlap, while comparable curves for PP-1 should reveal a 100-fold difference between the inhibitors.

Dose inhibition characterization of the phosphatase activity in DEAE peak fractions revealed that the activity in the first peak possessed IC_{50} curves characteristic of PP-1 (Fig 4.6 B) while the activity in the later eluting peak exhibited IC_{50} curves characteristic of PP-2A (Fig. 4.6C). Both of these peaks of activity were analyzed by Superdex-200 gel filtration chromatography. Those DEAE fractions containing phosphatase activity characteristic of PP-1 possessed an activity with an apparent native molecular mass of approximately 37 kDa (Fig. 4.6D), the molecular mass of the catalytic subunit of PP-1. PP-2A-containing fractions possessed an activity with an apparent native molecular mass of approximately 158 kDa (Fig. 4.6E). This mass corresponds well with the molecular mass of a native heterotrimeric complex of PP-2A [46]. In higher eukaryotes, this complex is composed of the catalytic dimer consisting of the 36 kDa catalytic subunit and 65 kDa regulatory subunit combined with a further regulatory subunit with an approximate molecular mass of 57 kDa. It is interesting to note that among higher eukaryotes the third regulatory subunit of PP-2A is a variable protein that confers different specificity to the PP-2A complex. My data suggest that PP-2A from *S. pombe* possesses similar quaternary structure to that of PP-2A from higher eukaryotes. It will be of interest to characterize the third regulatory subunit of *S. pombe* PP-2A to determine roles in which PP-2A regulates physiological processes in this unicellular eukaryote.

Fractions of a wild type *S. pombe* cell lysate eluted from DEAE weak anion exchange chromatography containing PP-1 activity were pooled and further resolved by Mono-Q strong anion exchange chromatography (Fig. 4.7A). Three peaks of phosphatase activity were identified in these fractions, termed peak I, II, and III according to their elution position. Since PP-2A activity might be present in the pooled DEAE fractions, the identity of the phosphatase activity in each peak was determined employing the marine toxins microcystin-LR and okadaic acid. The activity in peaks II and III (Fig. 4.7B) demonstrate toxin inhibition curves typical of PP-1 activity, where the IC_{50} of okadaic acid is 100-fold greater than that of microcystin. However, the activity eluting in peak I was resistant to both microcystin and okadaic acid (Fig. 4.6B), confounding its identity as either PP-1 or PP-2A. This enzyme is referred to herein as PP-r (protein phosphatase, resistant). In addition, this peak of phosphatase activity was resistant to PPIP46 (the active fragment of inhibitor-1 [47]), while the DEAE pool containing *S. pombe* PP-1 activity was sensitive to the inhibitor and the DEAE pool possessing *S. pombe* PP-2A activity was resistant, as expected (Fig. 4.8).

Recombinant human PP-1γ, native rabbit PP-2A, and PP-r from *S. pombe* were tested for their ability to be inhibited by PPIP46 (the active fragment of the PP-1-specific inhibitor-1), NaF (a nonspecific inhibitor of protein serine/threonine phosphatases),

Fig. 4.6 Characterization of phosphorylase phosphatase activity in wild type *S. pombe*. **A**. A wild type *S. pombe* cell lysate was separated by DEAE weak anion exchange chromatography and aliquots (20 µL) of the resulting fractions (5 mL) assayed for the presence of phosphorylase phosphatase activity, as outlined in the Materials and Methods section. The sensitivity of peak fractions 12 (B) and 22 (C) to the marine toxins microcystin-LR (closed circles) and okadaic acid (open circles) was determined. Fraction 12 manifested inhibition characteristic of PP-1 activity while fraction 22 contained PP-2A activity. Superdex-200 gel filtration analysis of fraction 12 (D) and 22 (E) revealed that fraction 12 contained an activity with an apparent native molecular mass of approximately 37 kDa, corresponding to the molecular mass of the catalytic subunit of PP-1. Fraction 22 possessed an activity with an apparent native molecular mass of approximately 158 kDa, which corresponds well with the molecular mass of a PP-2A heterotrimeric complex, as discussed in the text. The elution position of molecular mass standards is shown across the top of panels D and E. Dose response points represent averages of duplicate tests.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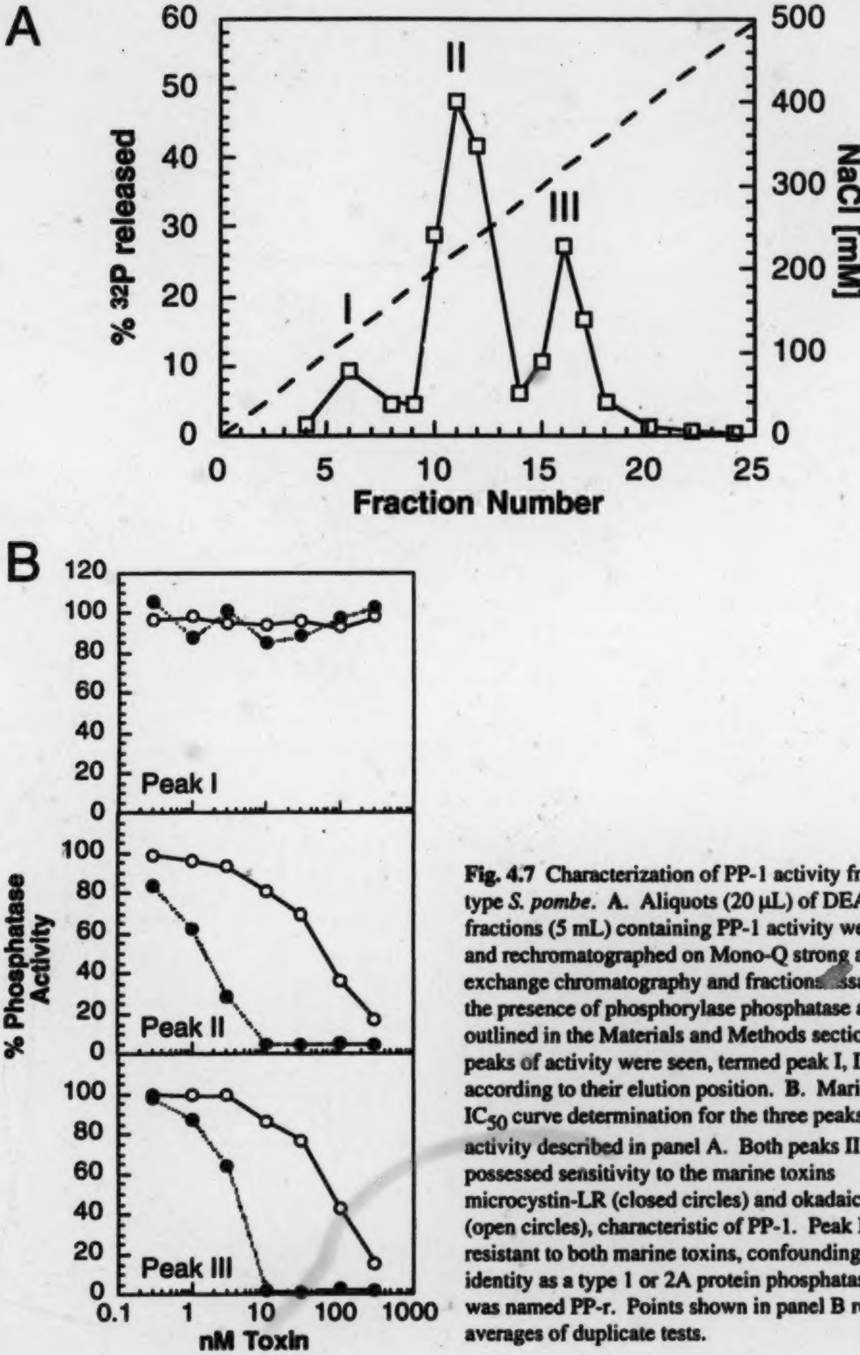


Fig. 4.7 Characterization of PP-1 activity from wild type *S. pombe*. A. Aliquots (20 µL) of DEAE fractions (5 mL) containing PP-1 activity were pooled and rechromatographed on Mono-Q strong anion exchange chromatography and fractions assayed for the presence of phosphorylase phosphatase activity as outlined in the Materials and Methods section. Three peaks of activity were seen, termed peak I, II, and III according to their elution position. B. Marine toxin IC_{50} curve determination for the three peaks of activity described in panel A. Both peaks II and III possessed sensitivity to the marine toxins microcystin-LR (closed circles) and okadaic acid (open circles), characteristic of PP-1. Peak I was resistant to both marine toxins, confounding its identity as a type 1 or 2A protein phosphatase, and was named PP-r. Points shown in panel B represent averages of duplicate tests.

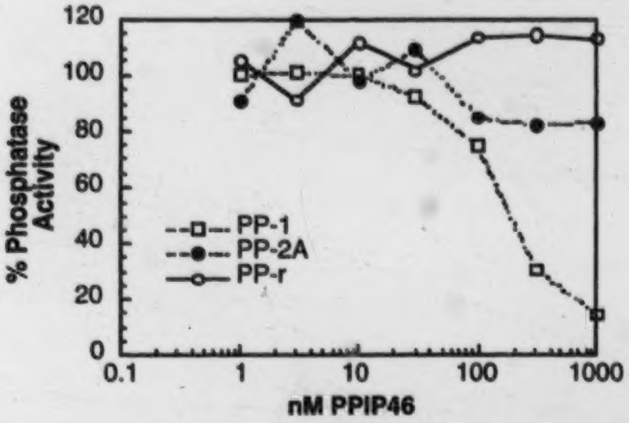


Fig. 4.8 Determination of the sensitivity of *S. pombe* phosphorylase phosphatase activities to PPIP46. PP-1 and PP-2A activities isolated by DEAE fractionation of a wild type *S. pombe* cell extract (see Fig. 4.6) and PP-r were tested for their sensitivity to PPIP46, the active fragment of the PP-1-specific inhibitor protein, inhibitor-1, discussed in section 1.4 (see Table 1.2). Phosphorylase was employed as substrate, as outlined in the Materials and Methods section. Only *S. pombe* PP-1 was sensitive to PPIP46. Data points shown represent the average of duplicate tests.

vanadate (a general protein tyrosine phosphatase inhibitor [48]), tartrate (an inhibitor of some acid phosphatases [49]), and tetramisole (an inhibitor of alkaline phosphatases [50]). Recombinant human PP-1γ was inhibited by only PPIP46 and NaF, while PP-2A from rabbit was inhibited only by NaF, as expected. PP-r activity was resistant to all the inhibitors tested, once again confounding the determination of its identity.

Gel filtration chromatography of the activity in peak I resulted in a broad peak of phosphatase activity eluting above 158 kDa, while the activities in both peak II and III eluted at a molecular mass corresponding to that of the catalytic subunit of PP-1 (37 kDa). These data suggest that *S. pombe* possesses a novel high molecular mass protein serine/threonine phosphatase that is resistant to all tested phosphatase inhibitors.

Characterization of PP-r

During large-scale preparation of native sds21 from Δdis2 *S. pombe* (producing only sds21) for PKA phosphorylation experiments, I found that the peak of activity eluting from DEAE corresponding to PP-1 in wild type *S. pombe* and rechromatographed on Mono-Q was completely resistant to microcystin and okadaic acid, identical to the PP-r activity characterized earlier. I suspected that PP-r activity was sds21 bound in a high molecular mass complex, but was unable to identify PP-r as a type-1 phosphatase because of its resistance to any inhibitors characteristic of PP-1.

Moreover, antisera raised against PP-1 from *S. pombe* (αD2C and αSds21, generous gifts of Dr. M. Yanagida, Kyoto University, Japan) and used in previous immunoprecipitation experiments [14,51,52], were found to crossreact with PP-2A from *S. pombe* in my hands. Hence, I could not rely on immunological data to identify the catalytic activity of PP-r.

Therefore, cell lysates from Δdis2 *S. pombe* (producing only sds21) and Δsds21 *S. pombe* (producing only dis2) were prepared and DEAE weak anion exchange chromatography employed to collect active fractions corresponding to the PP-1 peak of wild type cells. These pools were then rechromatographed by Mono-Q anion exchange chromatography (Fig. 4.9A). With each strain, two major peaks of activity were observed, labeled 1 and 2 according to their elution position. The activity in peak 1 from Δdis2 *S. pombe* was resistant to the toxins microcystin and okadaic acid (Fig. 4.9B) while its counterpart activity from Δsds21 *S. pombe* exhibited inhibition characteristic of PP-1 (Fig. 4.9C). However, the activity in peak 1 from Δsds21 *S. pombe* retained 15% activity at high toxin concentrations, demonstrating limited resistance to the toxins. The activity in peak 2 from both fission yeast strains was inhibited by the toxins microcystin and okadaic acid in a manner characteristic of PP-1 (Fig. 4.9B & C), although there was very little activity in this peak from Δdis2 *S. pombe*. The activity of the various peaks in the presence of PPIP46 and NaF was determined. The activity in peak 1 from Δdis2 *S. pombe* was resistant to both inhibitors, while the activities in all other peaks were inhibited by both compounds.

The elution positions of the phosphatase activities shown in Fig. 4.9A appear to be different from those for the wild type illustrated in Fig. 4.7A for two reasons. First, the number of DEAE fractions pooled and rechromatographed in the case of the knock-out strains were fewer than those pooled for the wild type *S. pombe* PP-1 purification and thus the activity in the peak corresponding to peak III of the wild type preparation is greatly reduced. Secondly, a newer Mono-Q column was employed for the purification of PP-1 from the knock-out strains of *S. pombe*. As a result, the proteins bound more tightly to the column and eluted at a higher salt concentration, delaying their fractionation. Thus, the two peaks of phosphatase activity observed in each of the knock-out strains and labeled 1 and 2 in Fig. 4.9A correspond to peaks I and II observed in the wild type (Fig. 4.7A).

Each of the peaks of activity shown in Fig. 4.9A was pooled and rechromatographed by Superdex-75 gel filtration chromatography. The pool of fractions containing peak 1 activity from both strains eluted at the void volume of the column,

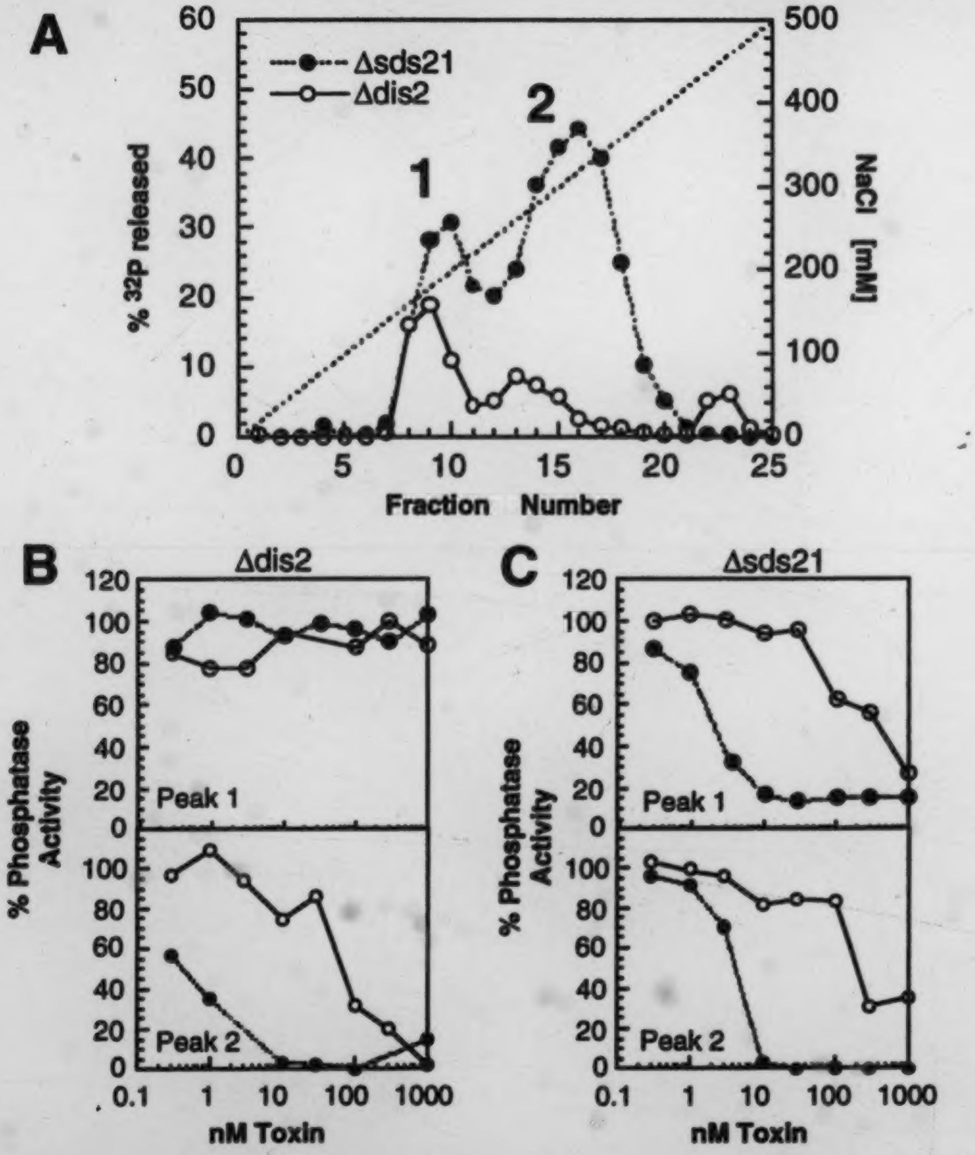


Fig. 4.9 Characterization of PP-1 activity from *S. pombe* PP-1 knock-out mutants. A. PP-1 activity identified from DEAE fractions of Δdis2 (open circles) and Δsds21 (closed circles) *S. pombe* was rechromatographed on Mono-Q and phosphorylase phosphatase activity assayed as described in the Materials and Methods section. Two peaks of activity were observed in each strain, termed peak 1 and 2 according to their elution position. B. Marine toxin IC_{50} curves employing microcystin-LR (closed circles) and okadaic acid (open circles) for the peaks from Δdis2 *S. pombe* illustrated that peak 1 was resistant to the toxins, characteristic of PP-r activity. Peak 2 from this strain demonstrated sensitivity characteristic of PP-1 activity, although the amount of activity in this peak was low. C. Both peaks from Δsds21 exhibited sensitivity to the marine toxins that were characteristic of PP-1 activity. Dose response points represent averages of duplicate tests.

suggesting a high molecular mass complex. The activity in the pool of fractions for peak 2 from Δdis2 *S. pombe* was too low to be detected in gel filtration fractions. However, the pool of fractions containing peak 2 activity from Δsds21 *S. pombe* eluted from Superdex-75 gel filtration chromatography with the molecular mass of free PP-1 catalytic subunit.

Given that both peak 1 activities from Δsds21 and Δdis2 *S. pombe* strains possess identical elution positions when analyzed by Mono-Q chromatography following DEAE chromatography and that they both consist of a high molecular mass phosphatase activity, it is probable that the phosphatase complex represented by peak 1 in each knock-out strain consists of the same subunit structure. The characterization of peak 1 from Δsds21 *S. pombe* demonstrating that it contains PP-1 activity (in this case, the dis2 isoform of PP-1) suggests that the comparable activity in peak 1 from Δdis2 *S. pombe*, which possesses all the characteristics of PP-r, must also contain a type-1 phosphatase catalytic subunit. In this case, however, the only type-1 phosphatase catalytic subunit expressed is sds21. Taken together, the evidence presented here strongly suggests that PP-r activity in *S. pombe* contains the sds21 isoform of PP-1.

In addition, the fact that the dis2-containing peak 1 does not possess significant amounts of free catalytic PP-1 activity, as seen in the gel filtration data, demonstrates that the high molecular mass complex is stable regardless of the *S. pombe* isoform of PP-1 that is present. The disparate inhibitor resistance of the two high molecular mass complexes is therefore most likely a product of the complex itself and not due to breakdown of the dis2-containing complex. Based on this evidence, the isoforms of PP-1 in *S. pombe* appear to be part of a high molecular mass complex which is resistant to inhibitors only when sds21 is present.

This dichotomy of inhibitor resistance between the two *S. pombe* high molecular mass PP-1 complexes is most likely based on structural differences between the two isoforms. In addition, structural differences may affect the formation of the complex. My data suggest that sds21 is preferred in the complex, since the amount of activity in Δdis2 *S. pombe* in the complex (peak 1) is greater than that observed for free sds21 subunit (peak 2). Conversely, the opposite is true in Δsds21 *S. pombe*. In this regard, the possession of a putative PKA phosphorylation site in sds21 that is lacking in dis2 may play a pivotal role.

Development of a Procedure for the Expression and Purification of Insoluble Protein Phosphatases in *E. coli*

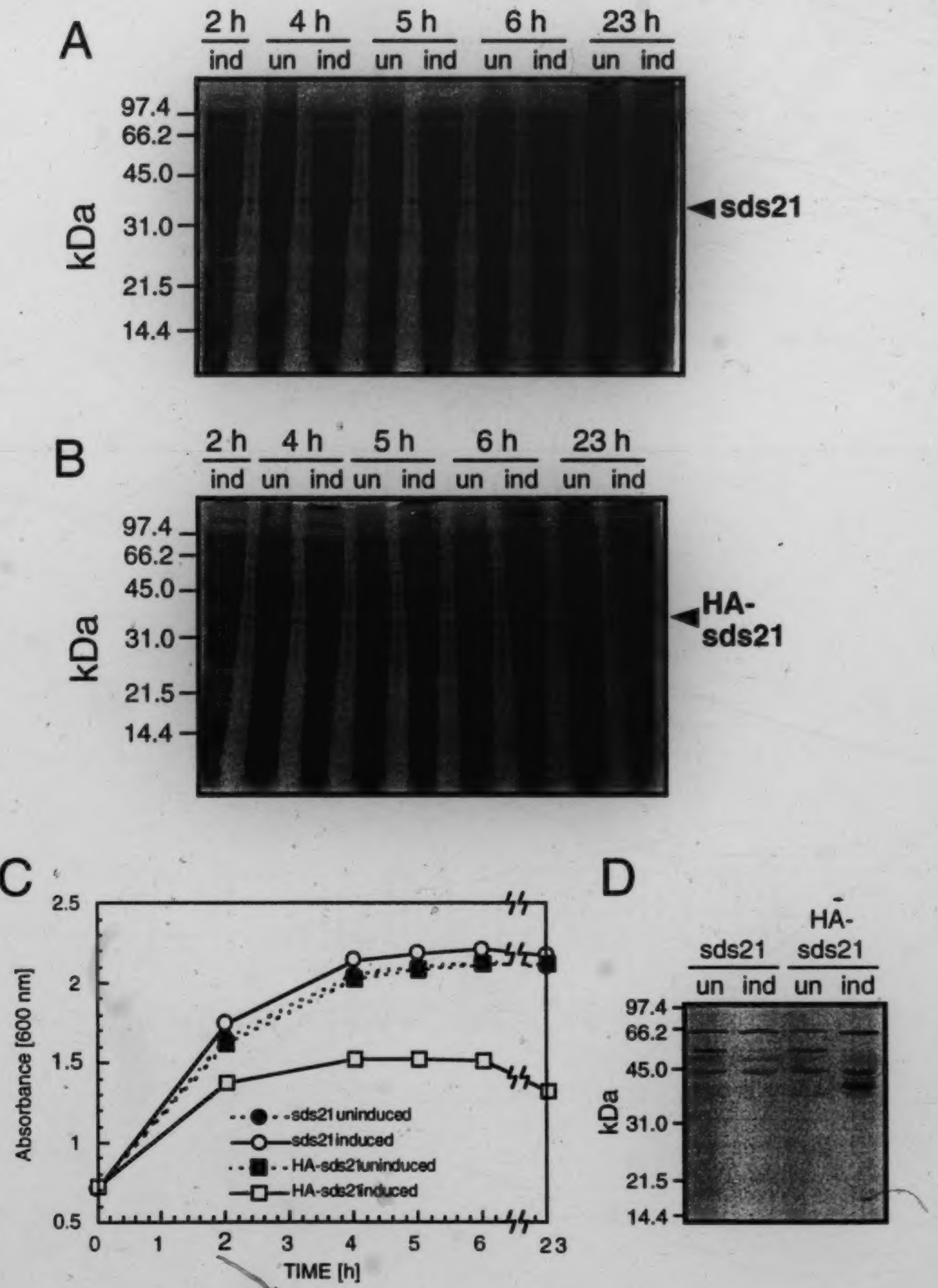
The purification of enough free sds21 to see a definable band on SDS-PAGE would have been ideal to test my hypothesis that it is a target of PKA. Unfortunately, I was unable to isolate enough free native sds21 from *S. pombe* to meet this criterion. I therefore obtained the cDNA encoding sds21 from Dr. M. Yanagida at Kyoto University in Japan so that recombinant sds21 might be expressed. The strategy employed to construct sequencing and expression vectors is outlined in Fig. 4.2. An immunoreactive epitope from influenza hemagluttinin protein recognized by the 12CA5 monoclonal antibody (often called an "HA-tag") was fused to the amino-terminus of sds21 in anticipation of future experiments involving *in vivo* immunoprecipitation of HA-tagged sds21 expressed in *S. pombe*. The choice of the amino terminus as the site of the HA-tag was based on earlier studies in which structurally related PP-2A was tagged at the amino- and/or carboxy-termini [53] and only amino-terminally tagged PP-2A was recognized by the 12CA5 antibody.

Unmodified and HA-tagged sds21 were expressed in DH-5α *E. coli* employing the pCW expression vector (Fig. 4.10). Induction of the expression of HA-tagged sds21 in *E. coli* resulted in inhibition of growth of the host cells. However, when cell lysates of uninduced and induced *E. coli* cells harbouring the sds21 or HA-tagged sds21 cDNA were run on SDS-PAGE, transferred to nitrocellulose and immunoblotted with the 12CA5 monoclonal antibody that recognizes the HA-affinity tag, an immunoreactive band corresponding to the molecular mass of sds21 was only present when the HA-tagged sds21 was expressed. Since expression of HA-tagged sds21 was toxic to the host *E. coli* cells, I elected to proceed with the purification of only unmodified sds21 protein.

Unlike human PP-1γ, which has been expressed in *E. coli* in soluble form [54], sds21 was insoluble when expressed in DH-5α. As a result, the purification protocols designed for the isolation of soluble recombinant PP-1 could not be applied to the purification of recombinant sds21. I therefore combined aspects of three protocols to develop my own protocol for the purification of sds21 from inclusion bodies. These included 1) FPLC experience with the purification of native PP-1 from rabbit skeletal muscle, which involves the step-wise elution of PP-1 from polylysine affinity chromatography, 2) the published purification procedure for recombinant soluble human PP-1γ [54], which involves Heparin-Sepharose affinity chromatography, and 3) the documented renaturation protocol for overexpressed mammalian PP-1 in the baculovirus system [39].

Insoluble sds21 was isolated from *E. coli* cells and solubilized in buffer containing guanidinium chloride. The protein was then renatured by dilution in buffer containing manganese and a high concentration of salt. The renaturation reaction mixture was then dialyzed to reduce the concentration of salt in the solution and the resulting dialysate was loaded onto a Heparin-Sepharose affinity chromatography column. Renatured soluble sds21 was then eluted using a step-wise increase in salt concentration in the running buffer and detected employing phosphorylase as substrate. The progress of purification of sds21

Fig. 4.10 Expression of sds21 in *E. coli*. **A.** DH-5α cells harbouring the pCW-sds21 plasmid (see the Materials and Methods section) were grown until an optical density of 0.5 to 0.6 was reached. Cells were then induced with the addition of 0.5 mM IPTG and samples of cells, indicated by (ind), were taken at time points indicated across the top. Samples from a parallel uninduced (un) culture were taken at the same time points. Whole cell lysates (10 μL) of these samples were run on SDS-PAGE (12%) and the proteins visualized by Coomassie blue staining. Induction of the production of sds21 was clearly seen. **B.** Same as panel A save that the pCW-HAsds21 plasmid was induced. **C.** Cell growth was monitored as a function of the optical density of a culture at 600 nm absorbance. Cell growth was inhibited in cultures in which the expression of HA-tagged sds21 was induced. **D.** Antigenicity of HA-tagged sds21 expressed in *E. coli*. Whole cell lystates (10 μL) of uninduced (un) and induced (ind) DH-5α cells harbouring the pCW-sds21 or pCW-HAsds21 plasmid were run on SDS-PAGE (12%), transferred to nitrocellulose and immunoblotted with the 12CA5 monoclonal antibody. A strong reacting band was observed only in lysates from induced cells with the pCW-HAsds21 plasmid with a molecular mass corresponding to sds21.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97.4
66.2
45.0
31.0
21.5
14.4
kDa
HA-sds21
C
Absorbance [600 nm]
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
23
TIME [h]
sds21 uninduced
sds21 induced
HA-sds21 uninduced
HA-sds21 induced
D
sds21
HA-sds21
un
ind
un
ind
97.4
66.2
45.0
31.0
21.5
14.4
kDa

from inclusion bodies is illustrated in Fig. 4.11. As a negative control, I also constructed a mutant form of sds21 in which the putative phosphoacceptor Ser-145 was substituted with an alanine residue that can not become phosphorylated. The progress of purification of this mutant is described in Appendix B.

With the simple protocol developed, good yields of wild type and mutant sds21 were obtained. The preparation was approximately 95% pure, with a small amount of contaminating protein with an apparent approximate molecular mass of 43 kDa being present. These preparations were then used in PKA phosphorylation reactions to test the hypothesis that sds21 is phosphorylated by PKA (see below).

Expression and Purification of sds21 in *S. pombe*

Investigating PP-1 activity in *S. pombe* afforded the opportunity to overexpress the *S. pombe* sds21 isoform of PP-1 in *S. pombe* itself. Since HA-tagged sds21 was toxic to DH-5α *E. coli* host cells, it was of interest to determine if expression of HA-tagged sds21 had a similar effect upon *S. pombe* cells. Ideally, sds21 would be expressed in Δdis2 *S. pombe* cells such that sds21 is the only isoform of PP-1 present in these cells. However, several attempts to transform Δdis2 *S. pombe* failed to produce any viable colonies. I therefore transformed Δsds21 *S. pombe* with sds21 or HA-tagged sds21 contained in the pREP-1 expression vector which utilizes the *nmt* repressor such that expression of sds21 occurs in the absence of thiamine.

To test the toxic effects of expressing HA-tagged sds21 in *S. pombe* cells, I patch-streaked *S. pombe* cells that had been transformed with pREP-1 containing either sds21 or HA-tagged sds21 on plates in the presence or absence of thiamine (Fig. 4.12). In the presence of thiamine, the expression of the sds21 constructs is repressed and cell growth occurs. In the absence of thiamine, expression of the sds21 constructs occurs. In this case, no noticeable inhibition of cell growth was observed when HA-sds21 was produced. Thus, expression of HA-tagged sds21 in *S. pombe* had no toxic effects on cellular growth, unlike the scenario with *E. coli*. This was an encouraging result, suggesting that HA-tagged sds21 could be expressed and purified from *S. pombe*.

Unmodified sds21 overexpressed in *S. pombe* was purified by sequential chromatographic steps on DEAE weak anion exchange, Source Q-15 strong anion exchange, and Superdex-75 gel filtration (Fig. 4. 13). sds21 purified in this fashion was tested for sensitivity to inhibition by microcystin-LR and okadaic acid (Fig. 4.13D). Overexpressed sds21 was inhibited by the marine toxins in a manner characteristic of PP-1.

Likewise, HA-tagged sds21 overexpressed in *S. pombe* was purified employing the same purification protocol outlined above (Fig. 4.14). In addition, HA-tagged sds21

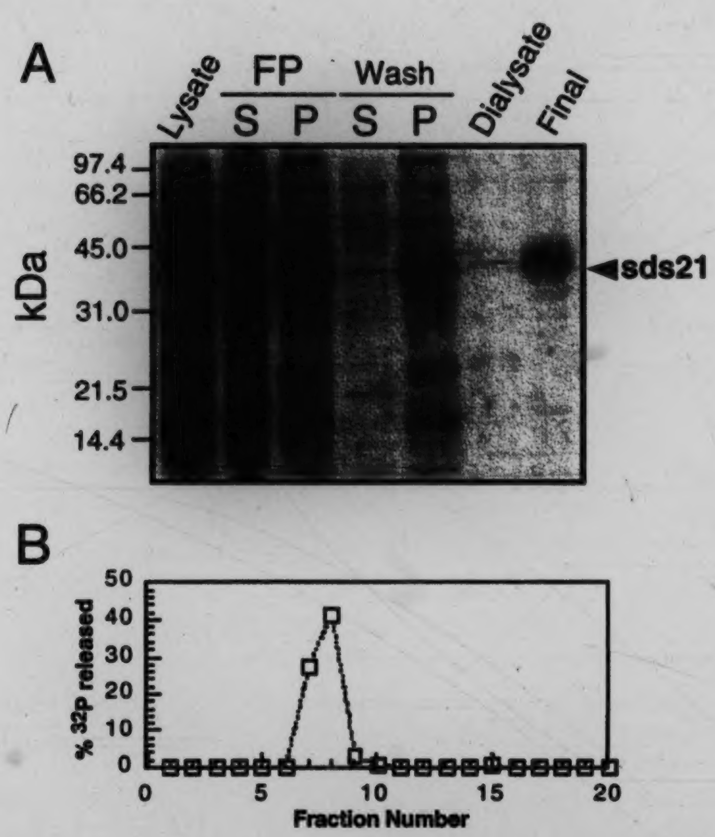


Fig. 4.11 Purification of sds21 from insoluble inclusion bodies in *E. coli*. **A.** Induced *E. coli* cell lysates (Lysate) produced employing a french press (FP) were centrifuged and the resulting pellet (P) separated from the supernatant (S). sds21 is present almost exclusively in the insoluble pellet fraction. This pellet was then washed (Wash) in a Triton X-100 containing buffer and the supernatant (S) separated from the pellet (P). This pellet was solubilized and renatured and the resulting renaturation reaction dialyzed to remove salt. The dialysate (Dialysate) was loaded onto Heparin-Sepharose and sds21 eluted. Active fractions were pooled and dialyzed against storage buffer to yield the final protein preparation (Final). Aliquots (5 µL, save the Lysate (10 µL) and Dialysate (20 µL)) of each stage of purification were run on SDS-PAGE (12%) and the proteins visualized by Coomassie staining.**B.** Detection of sds21 activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing isotopically labelled phosphorylase as substrate, as described in the Materials and Methods section. In this purification, 1/100th of 1 µL of each fraction (5 mL) was assayed. The first five fractions were run in buffer containing 200 mM salt. The salt concentration was then increased to 450 mM for the next ten fractions and finally to 600 mM for the final five fractions. sds21 eluted in the lead fractions containing 450 mM salt.

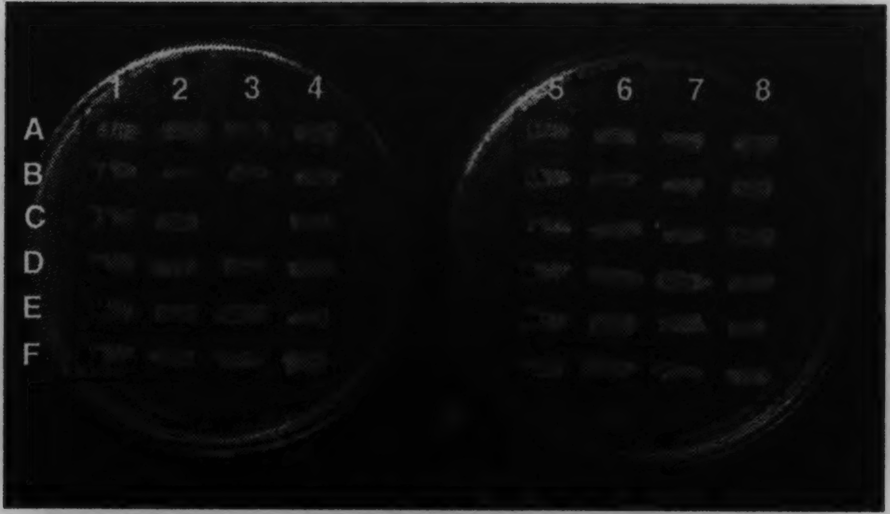


Fig. 4.12 Expression of sds21 and HA-tagged sds21 in Δsds21 *S. pombe*. Six different pREP-1 constructs (A to F) were transformed into Δsds21 *S. pombe* and four different transformants of each construct were patch-streaked onto plates in the absence (1 to 4) or presence (5 to 8) of thiamine. The six pREP-1 constructs were: **A**. pREP-1-sds21. **B**. pREP-1-S145Asds21. **C**. pREP-1-S145Dsds21, a mutation of the putative phosphoacceptor serine to aspartic acid which was not characterized further. **D**. pREP-1-HAsds21. **E**. pREP-1-HA-S145Asds21. **F**. pREP-1-HA-S145Dsds21, the same aspartic acid subsitution mutant described above with an HA-tag that was not characterized further. In the presence of thiamine (5 to 8), the *nmt* promoter of pREP-1 was repressed and no sds21 protein is expressed. In this case, normal cell growth was observed. In the absence of thiamine (1 to 4), expression of the sds21 constructs occurred. No inhibition of growth was observed in *S. pombe* cells expressing HA-tagged sds21, contrary to the case in *E. coli* (see Fig. 4.9C).

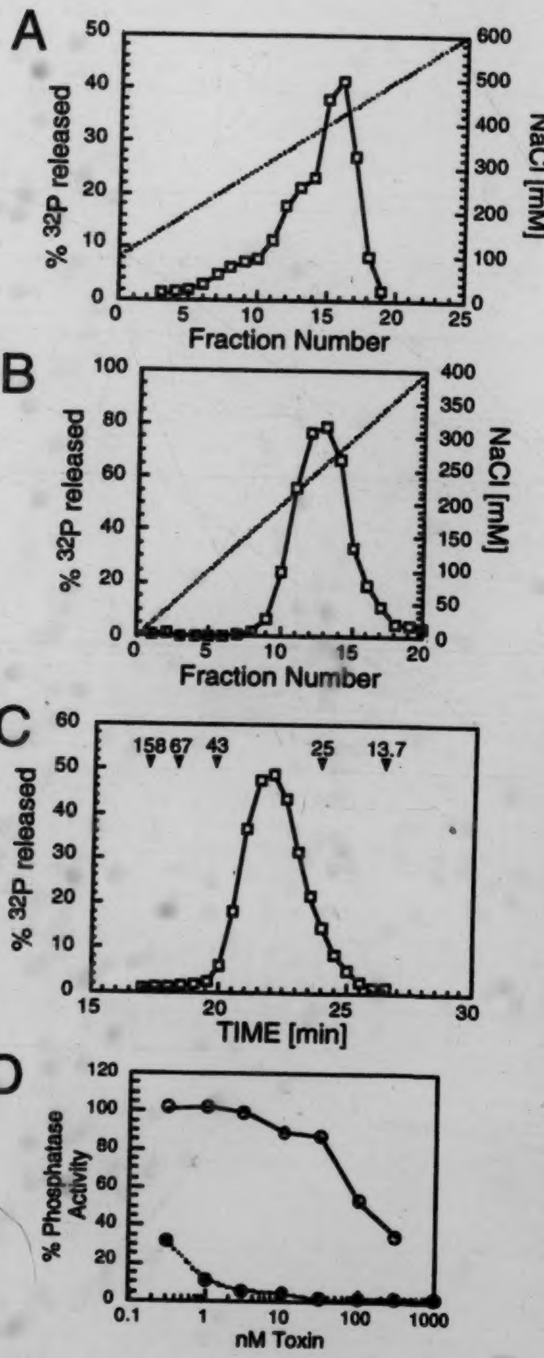


Fig. 4.13 Purification of sds21 overexpressed in *S. pombe*. **A.** A cell lysate of unrepressed *S. pombe* cells harbouring the pREP-1-sds21 plasmid was loaded onto Heparin-Sepharose and phosphorylase phosphatase activity assayed in 10 µL of the resulting 5 mL fractions, as described in the Materials and Methods section. **B.** Active Heparin-Sepharose fractions were pooled and rechromatographed on Source Q-15. 5 µL of each 1 mL fraction was assayed for phosphatase activity. **C.** Active Source Q-15 fractions were concentrated and run on Superdex-75 gel filtration chromatography.2 µL of each 250 µL fraction was assayed. Active fractions were pooled and dialyzed against storage buffer prior to storage at -20 °C. **D.** IC_{50} curves for purified sds21 overexpressed in *S. pombe* for the marine toxins microcystin-LR (closed circles) and okadaic acid (open circles). These curves are characteristic of a type-1 protein phosphatase activity. Dose response points represent averages of duplicate tests.

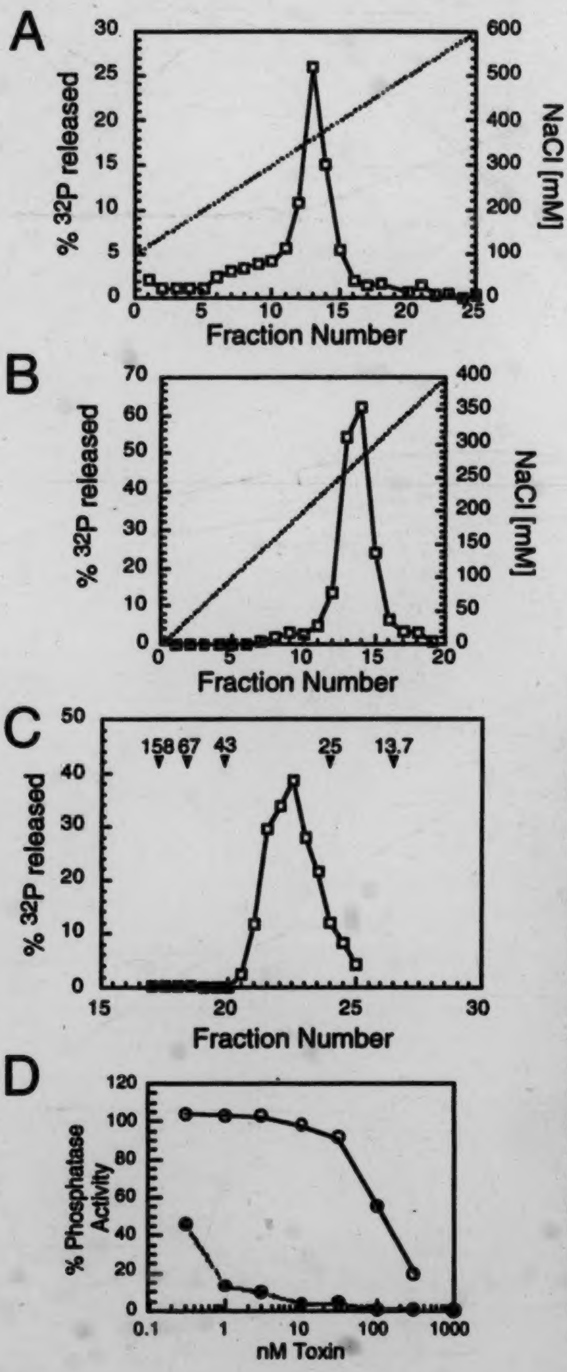


Fig. 4.14 Purification of HA-tagged sds21 overexpressed in *S. pombe*. **A.** A cell lysate of unrepressed *S. pombe* cells harbouring the pREP-1-HAsds21 plasmid was loaded onto Heparin-Sepharose and phosphorylase phosphatase activity assayed in 10 µL of the resulting 5 mL fractions, as described in the Materials and Methods section. **B.** Active Heparin-Sepharose fractions were pooled and rechromatographed on Source Q-15. 5 µL of each 1 mL fraction was assayed for phosphatase activity. **C.** Active Source Q-15 fractions were concentrated and run on Superdex-75 gel filtration chromatography. 2 µL of each 250 µL fraction was assayed. Active fractions were pooled and dialyzed against storage buffer prior to storage at -20 °C. **D.** IC_{50} curves for purified sds21 overexpressed in *S. pombe* for the marine toxins microcystin-LR (closed circles) and okadaic acid (open circles). These curves are characteristic of a type-1 protein phosphatase activity. Dose response points represent averages of duplicate tests.

was assayed in the presence of the inhibitors microcystin-LR and okadaic acid (Fig. 4.14D) and found to be inhibited in a fashion characteristic of PP-1. Thus, the addition of the nine amino acids constituting the HA-tag to the amino-terminal of sds21 does not alter inhibition of the modified sds21 by the marine toxins.

Antigenicity of HA-tagged sds21 Expressed in *S. pombe*

Although the expression of HA-tagged sds21 in *E. coli* cells had negative effects on cell growth, the protein produced was still recognized by the 12CA5 monoclonal antibody (see Fig. 4.10D). It was important to establish that HA-tagged sds21 purified from *S. pombe* was also recognized by the antibody as a preliminary step to future experiments involving immunoprecipitation. Such experiments might be designed to examine possible sds21 *in vivo* interactions with other proteins regulated by the putative phosphorylation of the catalytic subunit by PKA.

Initially, Western immunoblotting of purified sds21 and HA-tagged sds21 failed to produce an immunoreactive band corresponding to HA-tagged sds21. To rule out the possibility that the antibody was not reacting properly, cell lysates of uninduced and induced *E. coli* harbouring the HA-tagged sds21 in the pCW expression vector were run on SDS-PAGE alongside purified sds21 and HA-tagged sds21 from *S. pombe*, transferred to nitrocellulose and immunoblotted with the 12CA5 monoclonal antibody (Fig. 4.15). A band corresponding to HA-tagged sds21 expressed in *E. coli* is readily seen on this immunoblot. However, HA-tagged sds21 purified from *S. pombe* was not immunoreactive. Thus, although expression of HA-tagged sds21 is not toxic to *S. pombe*, it is not antigenic.

Is sds21 Phosphorylated by PKA?

To test the hypothesis that sds21 is phosphorylated by PKA, I included recombinant wild type and S145A mutant sds21 in PKA phosphorylation reactions and ran these on SDS-PAGE (Fig. 4.16). Low level phosphorylation of both wild type sds21 and S145A sds21 was observed in these assays. It has been demonstrated elsewhere that PKA also phosphorylates the dis2 phosphatase *in vitro* to very low stoichiometry [52]. Weak phosphorylation of sds21 and S145A sds21 was observed only when PKA was present and was not affected by the presence of 0.8 μM microcystin-LR in the assay. When [α-^{32}P]-ATP was included in the phosphorylation reactions, no radioactive signal corresponding to sds21 or PKA autophosphorylation was observed. This suggests that sds21 is phosphorylated by PKA at very low stoichiometry at a site other than the classical

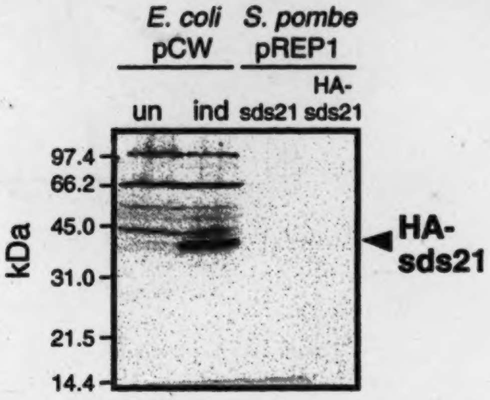


Fig. 4.15 HA-tagged sds21 overexpressed in *S. pombe* is not recognized by the 12CA5 monoclonal antibody. Uninduced (un) and induced (ind) whole cell lysates of DH-5α cells possessing the pCW-HAsds21 plasmid were run alongside sds21 and HA-tagged sds21 overexpressed and purified from *S. pombe*. HA-tagged sds21 produced in *E. coli* is antigenic while HA-tagged sds21 from *S. pombe* is not recognized by the 12CA5 monoclonal antibody.

PKA consensus sequence surrounding Ser-145. Thus, it appears that PKA does not phosphorylate Ser-145 of the *S. pombe* sds21 isoform of PP-1.

4.4 ■ DISCUSSION

Phosphorylation is a key mechanism of regulation of PP-1 activity in eukaryotic cells, as discussed in section 1.5. Not only is PP-1 activity modulated through phosphorylation of regulatory subunits that interact with PP-1, but the catalytic subunit of PP-1 is itself regulated by phosphorylation. The characterization of PKA activity in dinoflagellate cell extracts (see Fig. 3.11) suggested that PKA may influence the activity of a protein phosphatase, possibly PP-1 or PP-2A. In addition, sequencing of a cDNA fragment of PP-1 from the toxic marine dinoflagellate *Prorocentrum lima* revealed the presence of a classical PKA phosphorylation consensus sequence. This sequence is present among several lower eukaryotic PP-1 sequences (Table 4.1). Since I have not been able to detect inhibitor-1 activity in dinoflagellates, I hypothesized that PKA might regulate the activity of PP-1 directly through phosphorylation of the catalytic subunit, rather than through a polypeptide intermediate such as inhibitor-1 as is the case with higher eukaryotes. As such, phosphorylation of the catalytic subunit of PP-1 by PKA might represent a simpler signaling mechanism that evolved into a more complex system involving protein

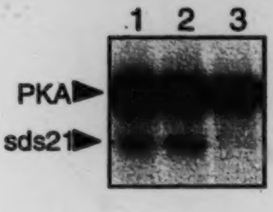


Fig. 4.16 sds21 is not phosphorylated by cAMP-dependent protein kinase on Ser-145 of the putative cAMP-dependent protein kinase phosphorylation site. PKA phosphorylation reactions containing 0.1 µg recombinant wild type sds21 (lane 1), 0.1 µg S145A sds21 (lane 2) and PKA alone (lane 3) were run on SDS-PAGE (12%) and an autoradiogram produced. A strong band of PKA auto-phosphorylation was seen. Both forms of sds21 exhibited low stoichiometric phosphorylation dependent on the presence of PKA, indicating that Ser-145 is not a target of PKA action.

intermediates in higher eukaryotes where coordinating multicellular growth is an added level of signaling complexity.

A peptide corresponding to the primary sequence surrounding the potential PKA phosphorylation site from *P. lima* PP-1 was effectively phosphorylated by PKA and dephosphorylated by calcineurin (PP-2B). The crystal structure of rat PP-1 (see Fig 1.10) suggests that the homologous region of the phosphatase from lower eukaryotes possessing the putative PKA phosphorylation site is located at the enzyme's surface at a turn between helix E and F, making it potentially accessible for phosphorylation by PKA *in vivo*.

I therefore attempted to purify PP-1 from the fission yeast *S. pombe*, since this is an easily manipulated system where only two isoforms of PP-1, one including the putative PKA phosphorylation site (sds21) and one not (dis2), are present. Initial purification of phosphorylase phosphatase activity in wild type *S. pombe* cell extracts revealed the presence of both PP-1 and PP-2A activities in fractions eluted from DEAE weak anion exchange chromatography. Subsequent separation of PP-1-containing DEAE fractions on Mono-Q strong anion exchange chromatography manifested three phosphatase activities, one of which was completely resistant to all relevant phosphatase inhibitors tested, which I termed PP-r. Since PP-r was resistant to all the inhibitors tested, it could not be positively identified as a PP-1 or PP-2A activity. However, these initial experiments with wild type cells demonstrated the ability to purify native PP-1 from *S. pombe*.

Attempts to purify native sds21 from Δdis2 *S. pombe* for use in PKA phosphorylation experiments were made. However, the activity purified corresponding to PP-1 activity in DEAE fractions and rechromatographed on Mono-Q strong anion exchange chromatography was completely resistant to the inhibitors tested, characteristic of PP-r activity. Parallel separation of Δdis2 or Δsds21 *S. pombe* cell extracts by DEAE and

Mono-Q chromatography resulted in two Mono-Q resolvable peaks of phosphorylase phosphatase activity, termed peak 1 and peak 2 according to their elution position. Peak 2 activity manifested inhibitor sensitivity characteristic of PP-1 in both Δdis2 and Δsds21 *S. pombe*. Peak 2 activity from *S. pombe* expressing dis2 eluted from gel filtration at a time corresponding to the free catalytic subunit of PP-1. Therefore, I concluded that the activity in this peak contains free dis2 catalytic subunit. Although there was not enough activity in the sds21-containing second peak to clearly observe phosphatase activity by gel filtration, I concluded based on comparable elution position on Mono-Q and inhibitor sensitivity that this peak represented free catalytic sds21.

Peak 1 activity represented a high molecular mass protein phosphatase activity in both knock-out strains of *S. pombe*. When only the dis2 isoform of PP-1 is expressed, as in the case of the Δsds21 strain, the activity from peak 1 was inhibited by the toxins microcystin-LR and okadaic acid in a manner characteristic of a type-1 phosphatase. In addition, peak 1 activity in Δsds21 *S. pombe* was sensitive to inhibition by PPIP46 and NaF, also characteristic of PP-1 activity. This suggests that in the Δsds21 strain, the high molecular mass phosphatase observed in peak 1 contains the dis2 isoform of PP-1 as the catalytic subunit.

Conversely, when only the sds21 isoform of PP-1 was expressed in *S. pombe*, as in the case of the Δdis2 strain, the activity observed in peak 1 was resistant to the toxins microcystin-LR and okadaic acid, characteristic of PP-r activity observed earlier in wild type cells. Moreover, peak 1 activity in Δdis2 *S. pombe* was resistant to inhibition by PPIP46 and NaF, again characteristic of PP-r activity. Therefore, I conclude that peak 1 from Δdis2 *S. pombe* contains PP-r phosphatase activity.

Given that the peak 1 activities from both the Δsds21 and Δdis2 possess similar elution positions off Mono-Q chromatography following DEAE chromatography and that they both consist of a high molecular mass phosphatase activity, it is probable that both complexes contain the same subunit structure. Since peak 1 activity from Δsds21 *S. pombe* contained the dis2 PP-1 catalytic subunit, the comparable activity in peak 1 from Δdis2 *S. pombe* most likely also contains PP-1. In this case however, the only catalytic subunit of PP-1 expressed was sds21. Therefore, I conclude that the activity in peak 1 from Δdis2 *S. pombe* contains the sds21 type-1 phosphatase. Since peak 1 from Δdis2 *S. pombe* contains PP-r activity initially characterized in wild type cells, I conclude that PP-r activity contains the sds21 isoform of PP-1.

The fact that the dis2-containing peak 1 from Mono-Q chromatography did not possess significant free catalytic dis2 demonstrates that the high molecular mass complex is stable regardless of the isoform of PP-1 catalytic subunit it binds. Therefore, there exist two high molecular mass PP-1 complexes in *S. pombe* that have differing inhibitor sensitivity and possess different PP-1 catalytic subunits. There are several scenarios that might explain this. First, each isoform of PP-1 in *S. pombe* may bind to different complex proteins which confer differing inhibitor sensitivity to the catalytic subunit. Alternatively, the different PP-1 catalytic subunit isoforms may possess differential inhibitor sensitivity when bound in the same complex. In either case, the different inhibitor sensitivity of the complexes is most likely due to structural differences between the sds21 and dis2 catalytic subunits. In this regard, the possession of a cdc2 phosphorylation site on the C-terminal of dis2 [52] and/or a putative PKA phosphorylation site on sds21 may play a crucial role.

I was unable to purify enough native free sds21 to test the hypothesis that it is phosphorylated by PKA. Therefore, I expressed two forms of sds21 in *E. coli*, one that possesses an amino-terminal modification consisting of one copy of the nine amino acid hemagluttinin epitope tag (HA-tag) and one that does not. It was observed that expression of HA-tagged sds21 in *E. coli* caused inhibition of cellular growth. However, HA-tagged sds21 expressed in *E. coli* was immunoreactive with the 12CA5 monoclonal antibody which recognizes the HA-tag. Unfortunately, since cellular growth was inhibited, HA-tagged sds21 was not purified from *E. coli*. In addition to wild type sds21, a substitution mutant in which the putative serine phosphoacceptor residue was changed to a non-phosphorylatable alanine residue was constructed.

Unlike human PP-1γ, which is expressed in soluble form in *E. coli*, sds21 was expressed in insoluble form in *E. coli*. Therefore, I developed a protocol for purifying sds21 from inclusion bodies involving renaturation of solubilized sds21 and purification on Heparin-Sepharose chromatography. This protocol was very convenient for the purification of sds21. A typical purification took only four days from single *E. coli* colony to purified protein: two days to grow and induce the *E. coli* host cells, one day to produce the cell lysate, solubilize and renature sds21, and a final day to purify the soluble sds21 by Heparin Sepharose affinity chromatography.

This protocol can be applied to any form of PP-1 that is expressed in insoluble form in *E. coli* and would prove to be invaluable in future work outlined in Chapter 5 where multiple mutant forms of human PP-1γ were purified. The major drawback of this protocol is that purification of large amounts of PP-1 is challenging due to the large volume of the renaturation reaction. Typically, 100 mL of culture is used and insoluble PP-1 solubilized in a total volume of 4 mL buffer containing guanidinium chloride (see Materials and Methods). Renaturation occurs by diluting the solubilized protein solution in 500 mL of buffer containing manganese and a high concentration of salt.

The main obstacle to larger scale purifications is the reduction of the concentration of salt in the renaturation reaction such that the solubilized and renatured PP-1 can be loaded onto Heparin-Sepharose. I chose to dialyze the renaturation reaction overnight against buffer containing no salt, rather than further dilute the renaturation reaction, as was carried out by Berndt and Cohen in their protocol for purification of insoluble PP-1 expressed in the baculovirus system [39]. In order to achieve an acceptable salt concentration for Heparin-Sepharose chromatography, a 500 mL renaturation reaction must be diluted to 10 L. Although Heparin-Sepharose is a fast-flow chromatographic resin, with loading rates of 5 mL/min possible, constantly loading 10 L at this rate would still require 33.3 hours! As the salt concentration decreases over the course of the dialysis, aggregation of PP-1 occurs in the dialysis tubing, resulting in the loss of some protein. However, enough PP-1 remains soluble such that a significant amount can be purified, and loading 500 mL onto Heparin-Sepharose only requires 1 hour and 40 minutes. Thus, loss of protein that occurs during dialysis was acceptable to expediate the purification of insoluble recombinant PP-1.

Investigation of PP-1 isoforms from *S. pombe* affords the possibility of overexpressing the proteins in *S. pombe* itself, circumventing debate regarding differences between recombinant and native enzymes. In addition, since HA-tagged sds21 inhibited growth when expressed in *E. coli*, it was important to determine if the same effect occurred when HA-tagged sds21 was expressed in *S. pombe*. Moreover, it was important to determine if HA-tagged sds21 expressed in *S. pombe* was antigenic, since HA-tagged sds21 could be a potentially powerful tool for delineating *in vivo* protein-protein interactions that might be regulated by the potential phosphorylation of sds21 by PKA using immunoprecipitation techniques.

Expression of HA-tagged sds21 in *S. pombe* was found to cause no apparent inhibition of cell growth. Wild type and HA-tagged sds21 overexpressed in *S. pombe* cells were subsequently purified. Both forms of sds21 were inhibited by the marine toxins microcystin-LR and okadaic acid in a manner characteristic of PP-1. However, it was discovered that HA-tagged sds21 isolated from *S. pombe* was not recognized by the 12CA5 monoclonal antibody, in contrast to HA-tagged sds21 expressed in *E. coli* cells. It has been noted that in the budding yeast *Saccharomyces cerevisiae* a triple copy of the HA-tag was much more antigenic than a single copy of the epitope tag [55]. As a result, expression vectors for *S. pombe* have been constructed that possess a triple copy of the HA-tag [56]. The HA-tagged version of sds21 that I constructed possessed only a single copy of the epitope tag and therefore might not be recognized by the monoclonal antibody. It will be important to determine if adding a triple tag to the amino-terminal of sds21 will affect the enzyme's activity and if such an enzyme will be readily recognized by the 12CA5 antibody if immunoprecipitation experiments aimed at investigating protein-protein interactions involving sds21 are desired in the future.

To test the hypothesis that PP-1 from lower eukaryotes is phosphorylated by PKA, wild type or S145A substitution mutant sds21 was included in PKA phosphorylation reactions. It was discovered that low-stoichiometric phosphorylation of both the wild type and mutant sds21 occurred in the presence of PKA. Therefore, it appears that sds21 is not a target of PKA at the classical consensus sequence identified earlier. This is in contrast to the phosphorylation and dephosphorylation of a fragment of PP-1 from the marine dinoflagellate *P. lima* surrounding the putative phosphorylation site. Closer inspection of the crystal structure of PP-1 from rat [57] revealed that, although the region homologous to the putative PKA phosphorylation site was solvent exposed and therefore presumably available to PKA, its conformation is highly constrained as it lies in a hair-pin turn between α-helix 5 and 6 (Fig. 4.17).

The molecular mechanism of substrate binding by PKA has been extensively discussed since the determination of the crystal structure of PKA bound to an active peptide fragment of an endogenous pseudosubstrate inhibitor termed PKI [58]. Structural studies of PKA suggest that a phosphorylation consensus sequence in an extended conformation is most likely to be phosphorylated and that the two arginine residues of the PKA phosphorylation consensus sequence interact with acidic residues of the kinase in the substrate binding groove [59]. A comparison of the structure of PKI when bound to PKA and the region of sds21 surrounding the putative PKA phosphorylation site was performed employing the position of the two arginine residues of the consensus phosphorylation sequence as a common reference point. This comparison (Fig. 4.17B) reveals that, while the alanine of PKI (which would be the phosphoacceptor site in a PKA substrate) is oriented such that it would interact with the catalytic loop of the kinase, Ser-145 of sds21 is directed up and away from the active site of the kinase. Thus, it is suggested that the constrained conformation of the putative phosphorylation consensus sequence in the sds21 holoenzyme prevents it from being phosphorylated by PKA. Thus, regulation of lower eukaryotic isoforms of PP-1 by direct PKA phosphorylation does not appear to occur.

A potentially important finding from this work was the identification of PP-1 activities that exhibit different toxin sensitivities depending on the isoform of catalytic subunit they possess. Several groups have heralded microcystin-affinity chromatography as a means of identifying novel complexes of PP-1 [60,61]. I have identified a PP-1 complex that is resistant to inhibition by microcystin, presumably due to an inability of the toxin to bind to PP-1. Therefore, while microcystin-affinity chromatography has

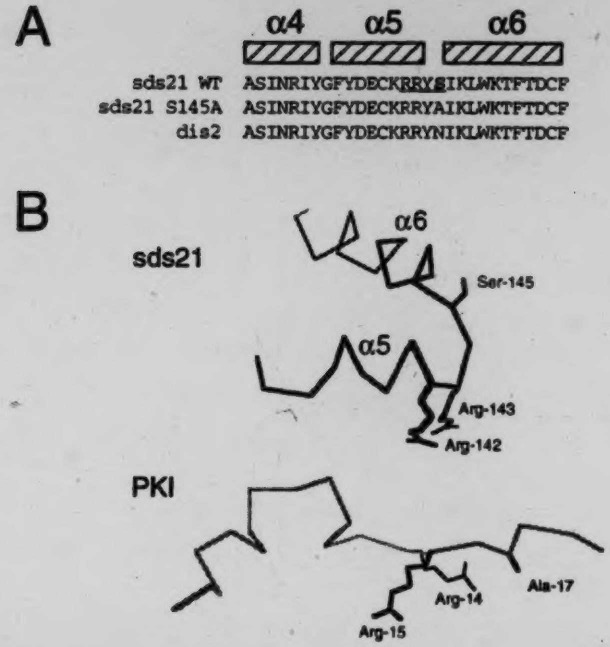

A
α4
α5
α6
sds21 WT ASINRIYGFYDECKRRYSIKLWKTFTDCF
sds21 S145A ASINRIYGFYDECKRRYAIKLWKTFTDCF
dis2 ASINRIYGFYDECKRRYNIKLWKTFTDCF
B
sds21
α6
Ser-145
α5
Arg-143
Arg-142
PKI
Ala-17
Arg-14
Arg-15

Fig. 4.17 The secondary and tertiary structure surrounding the putative cAMP-dependent protein kinase phosphorylation site on some isoforms of protein phosphatase-1. **A.** A sequence alignment of fragments of wild type sds21, the S145A mutant of sds21 and dis2 surrounding the putative PKA phosphorylation site on sds21 (underlined with phosphoacceptor site in bold). The secondary structure of the homologous sequence in the rat PP-1 crystal structure is indicated across the top. **B.** Comparison of the crystal structure of PKI, a pseudosubstrate inhibitor of PKA, when held in the PKA substrate binding groove (bottom) and the fragment of the rat PP-1 crystal structure surrounding the putative phosphoacceptor site (top). Coordinates for the Ser-145 indicated are arbitrary and are shown only to indicate the general position of the putative phosphoacceptor site.

successfully been employed to identify novel proteins that associate with PP-1, PP-1 complexes that fail to bind microcystin, such as the sds21-containing PP-r, would escape detection. Hence, microcystin-affinity chromatography must be utilized with the understanding that there may exist important forms of PP-1 that will not bind microcystin, as I have demonstrated. In *S. pombe*, such an inhibitor-resistant PP-1 could guard against the detrimental effects of toxic inhibitors in its environment that would upset the delicate balance of cellular protein phosphorylation.

It will be of interest to identify the interacting subunit(s) of the high molecular mass complex for structural and biochemical studies and to determine if there are homologous proteins in higher eukaryotes which may form similarly resistant forms of PP-1. A logical course of experimentation would be the identification of the different subunits contained in the high molecular mass sds21-containing PP-r complex to gain insight into the physiological roles played by PP-r in *S. pombe*. However, higher amounts of the complex are required for peptide mapping and microsequencing of the component complex proteins than that which I purified. In addition, two-hybrid isolation of *S. pombe* proteins that potentially interact with sds21 might result in the identification of several proteins, many of which have no bearing on the PP-r complex. Likewise, the use of microcystin affinity chromatography would fail to isolate PP-r, since it is resistant to this inhibitor, most likely due to the inability of the inhibitor to bind to the phosphatase. As a result, further characterization of PP-r was not carried out.

Although the hypothesis that PP-1 is phosphorylated by PKA among lower eukaryotes does not appear to be true, the work performed in this chapter laid the groundwork for the technical challenges involved in producing mutants of human PP-1γ to test the interactions of PP-1 and PP-2B with the marine toxins microcystin-LR, nodularin, and okadaic acid as outlined in the following Chapter. The insoluble PP-1 preparation protocol that I developed initially to purify sds21 proved to be an invaluable asset to our mutagenesis strategy for human PP-1γ discussed in Chapter 5.

4.5 ■ BIBLIOGRAPHY

1. Cohen, P., and Cohen, P. T. W. (1989) Protein phosphatases come of age. *J. Biol. Chem.* **264**: 21435-21438.

2. Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **59**: 453-508.

3. Bollen, M., and Stalmans, W. (1992) The structure, role, and regulation of type 1 protein phosphatases. *Crit. Rev. Biochem. Mol. Biol.* **27**: 227-281.

4. Mumby, M. C., and Walter, G. (1993) Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. *Physoil. Rev.* **73**: 673-699.

5. Carmichael, W. W. (1994) The toxins of cyanobacteria. *Sci. Am.* **270**: 78-86.

6. Murakami, Y., Oshima, Y., and Yasumoto, T. (1982) Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. *Bull. Jpn. Soc. Sci. Fish.* **48**: 69-72.

7. Cohen, P., Holmes, C. F. B., and Tsukitani, Y. (1990) Okadaic acid: a new probe for studying cellular regulation. *Trends Biochem. Sci.* **15**: 98-102.

8. Boland, M. P., Taylor, M. F. J. R., and Holmes, C. F. B. (1993) Identification and characterisation of a type-1 protein phosphatase from the okadaic acid producing marine dinoflagellate *Prorocentrum lima*. *FEBS Lett.* **334**: 13-17.

9. Boland, M. P., and Holmes, C. F. B. (1994) Identification and characterisation of protein phosphatase-1 and -2A in marine dinoflagellates. *Adv. Prot. Phosphatases* **8**: 325-342.

10. Zhou, J., and Fritz, L. (1994) Okadaic acid antibody localizes to chloroplasts in the DSP-producing dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum*. *Phycologia* **33**: 455-461.

11. Barker, H. M., Jones, T. M., da Cruz e Silva, O. B., da Cruz e Silva, E. F., Spurr, N. K., Sheer, D., and Cohen, P. T. W. (1990) Localization of the gene encoding a type 1 protein phosphatase catalytic subunit to human chromosome band 11q13. *Genomics* **7**: 159-166.

12. Cohen, P. T. W., Schelling, D. L., da Cruz e Silva, O. B., Barker, H. M., and Cohen, P. (1989) The major type-1 protein phosphatase catalytic subunits are the same gene products in rabbit skeletal muscle and rabbit liver. *Biochim. Biophys. Acta* **1008**: 125-128.

13. Berndt, N., Campbell, D. G., Caudwell, F. B., Cohen, P., da Cruz e Silva, E. F., da Cruz e Silva, O. B., and Cohen , P. T. W. (1987) Isolation and sequence analysis of a cDNA clone encoding a type-1 protein phosphatase catalytic subunit: homology with protein phosphatase 2A. *FEBS Lett.* **223**: 340-346.

14. Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T., and Yanagida, M. (1989) The fission yeast *dis2*$^+$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell* **57**: 997-1007.

15. Becker, W., Kentrup, H., Klumpp, S., Schultz, J. E., and Joost, H. G. (1994) Molecular cloning of a protein serine/threonine phosphatase containing a putative regulatory tetratricopeptide repeat domain. *J. Biol. Chem.* **269**: 22586-22592.

16. Dombradi, V., Axton, J. M., Brewis, N. D., da Cruz e Silva, E. F., Alphey, L., and Cohen, P. T. W. (1990) Drosophila contains three genes that encode distinct isoforms of protein phosphatase 1. *Eur. J. Biochem.* **194**: 739-745.

17. Barker, H. M., Brewis, N. D., Street, A. J., Spurr, N. K., and Cohen, P. T. W. (1994) Three genes for protein phosphatase 1 map to different human chromosomes: sequence, expression and localisation of protein serine/threonine phosphatase 1 beta. *Biochim. Biophys. Acta* **1220**: 212-218.

18. Sasaki, S., Shima, H., Kitagawa, Y., Irino, S., Sugimura, T., and Nagao, M. (1990) Identification of members of the protein phosphatase 1 gene family in the rat and enhanced expression of protein phosphatase 1α gene in rat hepatocellular carcinomas. *Jpn. J. Cancer Res.* **81**: 1272-1280.

19. Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J., and Nakano, T. (1994) Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J. Biol. Chem.* **269**: 30407-30411.

20. Norman, S. A., and Mott, D. M. (1994) Molecular cloning and chromosomal localization of a human skeletal muscle PP-1 gamma 1 cDNA. *Mamm. Genome* : 41-45.

21. Walker, D. H., Rempel, R., and Maller, J. L. (1993) Serine/threonine protein phosphatase PP-1 gamma catalytic subunit from *Xenopus laevis*. *EMBL database* : direct submission.

22. Smith, R. D., and Walker, J. C. (1993) Expression of multiple type 1 phosphoprotein phosphatases in *Arabidopsis thaliana*. *Plant Mol. Biol.* **21**: 307-316.

23. Rundle, S. J., and Nasrallah, J. B. (1992) Molecular characterization of type 1 serine/threonine phosphatases from *Brassica oleracea*. *Plant Mol. Biol.* **20**: 367-375.

24. Smith, R. D., and Walker, J. C. (1991) Isolation and expression of maize type 1 protein phosphatase. *EMBL database* : direct submission.

25. Pay, A., Pirck, M., Bogre, L., Hirt, H., and Heberle-Bors, E. (1994) Isolation and characterization of phosphoprotein phosphatase 1 from alfalfa. *Mol. Gen. Genet.* **244**: 176-182.

26. Wang, B., Chang, M., Chen, X., and Wu, R. (1995) Serine/threonine protein phosphatase PP-1 from *Oryza sativa* (rice). *EMBL database* : direct submission.

27. Zimmerlin, A., Jupe, S. C., and Bolwell, G. P. (1995) Molecular cloning of the cDNA encoding a stress-inducible protein phosphatase 1 (PP1) catalytic subunit from french bean (*Phaseolus vulgaris* L.). *Plant Mol. Biol.* **28**: 363-368.

28. Dombradi, V., Axton, J. M., Glover, D. M., and Cohen, P. T. W. (1989) Cloning and chromosomal localisation of *Drosophila* cDNA encoding the catalytic subunit

of protein phosphatase 1(alpha). High conservation between mammalian and insect sequences. *Eur. J. Biochem.* **183**: 603-610.

29. Menzel, D., Vugrek, O., Frank, S., and Elsner-Menzel, C. (1995) Protein phosphatase 2A, a potential regulator of actin dynamics and actin-based organelle motility in the green algae *Acetabularia. Eur. J. Cell. Biol.* **67**: 179-187.

30. Doonan, J. H., and Morris, N. R. (1989) The *bim*G gene of Aspergillus nidulans, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. *Cell* **57**: 987-996.

31. Erondu, N. E., and Donelson, J. E. (1991) Characterization of trypanosome protein phosphatase 1 and 2A catalytic subunits. *Mol. Biochem. Parasitol.* **49**: 303-314.

32. Friderich, G., Klumpp, S., Russell, C. B., Hinrichsen, R. D., Kellner, R., and Schultz, J. E. (1992) Purification, characterization and structure of protein phosphatase 1 from the cilia of *Paramecium tetraurelia. Eur. J. Biochem.* **209**: 43-49.

33. Kinoshita, N., Ohkura, H., and Yanagida, M. (1990) Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. *Cell* **63**: 405-415.

34. Rasmussen, C., and Garen, C. (1993) Activation of calmodulin-dependent enzymes can be selectively inhibited by histone H1. *J. Biol. Chem.* **268**: 23788-23791.

35. Holmes, C. F. B. (1991) Liquid chromatography-linked protein phosphatase bioassay; a highly sensitive marine bioscreen for okadaic acid and related diarrhetic shellfish toxins. *Toxicon* **29**: 469-477.

36. MacKintosh, C. (1993) in *Protein phosphorylation: a practical approach* (Hardie, D. G., ed), pp. 197-230, Oxford University Press, New York

37. Takai, A., and Mieskes, G. (1991) Inhibitory effect of okadaic acid on the *p*-nitrophenyl phosphate phosphatase activity of protein phosphatases. *Biochem. J.* **275**: 233-239.

38. Voet, D., and Voet, J. G. (1990) in *Biochemistry*, pp. 329-354, John Wiley & Sons, Toronto

39. Berndt, N., and Cohen, P. W. T. (1990) Renaturation of protein phosphatase 1 expressed at high levels in insect cells using a baculovirus vector. *Eur. J. Biochem.* **190**: 291-297.

40. Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. (1990) High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe. Nucl. Acids Res.* **18**: 6485-6489.

41. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-252.

42. Blumenthal, D. K., Takio, K., Hansen, R. S., and Krebs, E. G. (1986) Dephosphorylation of cAMP-dependent protein kinase regulatory subunit (type II) by calmodulin-dependent protein phosphatase. *J.Biol. Chem.* **261**: 8140-8145.

43. Caudwell, F. B., Hiraga, A., and Cohen, P. (1986) Amino acid sequence of a region on the glycogen-binding subunit of protein phosphatase-1 phosphorylated by cyclic AMP-dependent protein kinase. *FEBS Lett.* **194**: 85-90.

44. Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **252**: 4888-4894.

45. Dawson, J. F., Boland, M. P., and Holmes, C. F. B. (1994) A capillary electrophoresis-based assay for protein kinases and protein phosphatases using peptide substrates. *Anal. Biochem.* **220**: 340-345.

46. Mayer-Jaekel, R. E., and Hemmings, B. A. (1994) Protein phosphatase 2A - a 'ménage a trois'. *Trends Cell Biol.* **4**: 287-291.

47. Holmes, C. F. B., and Boland, M. P. (1993) Inhibitors of protein phosphatase-1 and -2A; two of the major serine/threonine protein phosphatases involved in cellular regulation. *Curr. Opin. Struct. Biol.* **3**: 934-943.

48. Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1988) Characterization of the major protein-tyrosine-phosphatase of human placenta. *J. Biol. Chem.* **263**: 6731-6737.

49. Van Etten, R. L. (1982) Human prostatic acid phosphatase: a histidine phosphatase. *Ann. N.Y. Acad. Sci.* **390**: 27-51.

50. Van Belle, H. (1972) Kinetics and inhibition of alkaline phosphatases from canine tissues. *Biochim. Biophys. Acta* **289**: 159-168.

51. Stone, E. M., Yamano, H., Kinoshita, N., and Yanagida, M. (1993) Mitotic regulation of protein phosphatases by the fission yeast sds22 protein. *Curr. Biol.* **3**: 13-26.

52. Yamano, H., Ishii, K., and Yanagida, M. (1994) Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus sequence and its potential role in cell cycle regulation. *EMBO J.* **13**: 5310-5318.

53. Wadzinski, B. E., Eisfelder, B. J., Peruski, L. F., Mumby, M. C., and Johnson, G. L. (1992) NH_2-terminal modification of the phosphatase 2A catalytic subunit allows functional expression in mammalian cells. *J. Biol. Chem.* **267**: 16883-16888.

54. Barford, D., and Keller, J. C. (1994) Co-crystallization of the catalytic subunit of the serine/threonine protein phosphatase 1 from human in complex with microcystin LR. *J. Mol. Biol.* **235**: 763-766.

55. Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992) The Cln3-cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. *EMBO J.* **11**: 1773-1784.

56. Forsburg, S. L., and Sherman, D. A. (1997) General purpose tagging vectors for fission yeast. *Gene* **191**: 191-195.

57. Goldberg, J., Huang, H., Kwon, Y., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376**: 745-753.

58. Kemp, B. E., Parker, M. W., Hu, S., Tiganis, T., and House, C. (1994) Substrate and pseudosubstrate interactions with protein kinases: determinants of specificity. *Trends Biochem. Sci.* **19**: 440-443.

59. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Xuong, N.-H., Taylor, S. S., and Sowadski, J. M. (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**: 414-420.

60. Moorhead, G., MacKintosh, R. W., Morrice, N., Gallagher, T., and MacKintosh, C. (1994) Purification of type 1 protein (serine/threonine) phosphatases by microcystin-Sepharose affinity chromatography. *FEBS Lett.* **356**: 46-50.

61. Campos, M., Fadden, P., Alms, G., Qian, Z., and Haystead, T. A. J. (1996) Identification of protein phosphatase-1-binding proteins by microcystin-biotin affinity chromatography. *J. Biol. Chem.* **271**: 28478-28484.

Chapter 5

Elucidation of the Molecular Mechanism of Inhibition of Protein Phosphatase-1 by Marine Toxins

5.1 ■ INTRODUCTION

Protein phosphatase-1 (PP-1) is one of only four major protein serine/threonine phosphatases responsible for regulating a myriad of critical biochemical processes in eukaryotic cells. Initially, the phosphatases were regarded as constitutively active "housekeeping" enzymes, while protein kinases provided the dynamic regulation of phosphorylation homeostasis in cells. However, we now know that protein serine/threonine phosphatases are indeed regulated by a host of different mechanisms and should therefore be considered a dynamic counterpart of the protein kinases, possessing similar importance in eukaryotic cells. Indeed, some might argue that the protein serine/threonine phosphatases are more important than the kinases, given that there are only four major classes of this enzyme, compared to the thousands of protein serine/threonine kinases believed to exist in the human genome [1].

As we have discussed in section 1.4, PP-1 activity is regulated by association with other proteins that serve to either inhibit its activity or target the catalytic subunit to defined intracellular compartments and thereby restrict the subset of substrates with which it can interact. The paradigm for this mechanism is the association of the PP-1 catalytic subunit with the glycogen-binding subunit (G subunit) of PP-1G, which targets PP-1 activity to glycogen particles where it dephosphorylates and alters the activity of glycogen synthase and phosphorylase kinase (discussed in section 1.3).

Associated with the mechanism of PP-1 targeting and inhibitory proteins is an additional layer of PP-1 regulation by reversible protein phosphorylation, discussed in section 1.5. For example, phosphorylation of the heat-stable inhibitor protein inhibitor-1 (I-1) activates this polypeptide as a specific inhibitor of PP-1 activity. Likewise, phosphorylation plays a crucial role in the regulation of PP-1 by association with the G-subunit. Phosphorylation of the G-subunit of PP-1G by PKA causes the dissociation of the G-subunit from the catalytic subunit of PP-1, thereby abrogating the compartmentalization effect on the substrate preferences for PP-1. Conversely, phosphorylation of the G-subunit of PP-1G by insulin stimulated protein kinase (ISPK) increases the ability of PP-1G to dephosphorylate its glycogen-bound substrates. We have already encountered studies of the phosphorylation of a peptide fragment of the G-subunit of PP-1G on multiple sites in Chapter 2, where capillary electrophoresis was shown to be capable of resolving PP-1G peptide phosphorylated by different protein kinases on different sites.

More recently, it has been demonstrated that regulation of PP-1 activity occurs through phosphorylation of Thr-320 of the catalytic subunit of PP-1 itself. Studies with both mammalian and yeast PP-1 have shown that a C-terminal site is phosphorylated by the cell cycle dependent cdc2 kinase and that this phosphorylation event regulates PP-1 activity during the cell cycle. Direct phosphorylation of the catalytic subunit of PP-1 from some lower eukaryotes by cAMP-dependent protein kinase (PKA) was examined in Chapter 4. These studies with the fission yeast PP-1 isoform sds21 demonstrated that PKA does not phosphorylate these lower eukaryotic forms of PP-1 on Ser-145.

Regulation of Protein Phosphatase-1 by Marine Toxins

In addition to association with regulatory subunits and reversible protein phosphorylation, PP-1 activity is also regulated by a growing family of marine toxins. Among these are the polyether fatty acid okadaic acid (see Fig. 1.14) produced by marine dinoflagellates and the cyclic peptides microcystin (see Fig. 1.15) and nodularin (see Fig. 1.16) produced by cyanobacteria in freshwater and marine environments, as discussed in section 1.6. These toxins are all potent inhibitors of PP-1 and PP-2A and strong tumour promoters. Both enzymes are inhibited equally by the cyclic peptide toxins (IC_{50} of 0.2 nM), while PP-2A is 100-fold more sensitive to okadaic acid than PP-1 (IC_{50} of 0.2 nM and 20 nM, respectively) (see Table 1.2). These toxins were employed in my work described in Chapter 4 to determine the identity of phosphorylase phosphatase activity in fission yeast cell extracts.

Given the extreme sensitivity of PP-1 to the marine toxins, it is often considered a cellular receptor of these toxins, providing an enzymatic basis for their toxicity. Since PP-1 is involved in so many vital physiological processes, it is important to understand the mechanism(s) of inhibition of PP-1 by the marine toxins. In Chapter 3, we tested the hypothesis that investigating signal transduction involving protein kinases and phosphatases in the marine dinoflagellate *Prorocentrum lima* that produces okadaic acid, might provide insight into how PP-1 activity is regulated by okadaic acid. I found that this unicellular organism possesses signal transduction molecules that are essentially the same as other unicellular eukaryotes. This countered the idea that *P. lima* does not exhibit any ill effects from the presence of okadaic acid due to its protein phosphatases being somehow different from those in animals that are affected by the toxin. This evidence also lends support to the hypothesis that dinoflagellates are true eukaryotes and are not of mesokaryotic origin.

Since the interaction of the marine toxins with PP-1 involves two players, past experimental strategies aimed at understanding the mechanisms of those interactions have been performed from two different reference points. One strategy has been to examine structure-function relationships between the toxins and the phosphatases from the context of the toxins. This strategy has the most historical precedent, since there are several naturally-occurring variations of microcystins and due to the ease of chemical modifications of all the marine toxins. More recently, the application of molecular biological techniques has permitted examination of the contribution of the phosphatase itself to the interaction with the marine toxins through site-directed mutagenesis and expression of recombinant enzyme. Moreover, the determination, in 1995, of the crystal structure of PP-1 with the marine toxin microcystin-LR bound has provided a clear structural picture of the interaction of the phosphatase with this cyclic peptide inhibitor.

Insights Derived from Structural Analysis of the Marine Toxins

Several groups have analyzed the ability of different structural variations of the marine toxins on their ability to inhibit phosphatases or to exert toxic effects. Initial work with okadaic acid demonstrated that modification of the carboxylic acid group of carbon-1 (C1, see Fig. 1.14) caused significant decreases in the ability of the toxin to inhibit phosphatase activity [2,3], such that okadaic acid with a methyl ester on C1 was completely ineffective as a phosphatase inhibitor. The same study demonstrated that methylation of the oxygen atoms associated with C2, 7, 24, and 27 also caused severe decreases in toxin effectiveness. This evidence suggests that the acid moiety of okadaic acid is of great importance and that the overall size of the molecule, which is increased by methylation of the four oxygen atoms, also plays a role in okadaic acid toxicity.

The isolation of naturally-occurring variations of microcystins has also provided clues as to the structural features of this cyclic peptide that are key to its interactions with PP-1. Most commonly, novel microcystins were tested in a mouse bioassay to determine the LD_{50} of the toxin (50% of the lethal concentration of toxin delivered intraperitoneally) [4,5]. The LD_{50} of microcystin-LR is approximately 50 µg/kg in mice. Since the binding and inhibition of phosphatases by microcystins form the likely basis of the effects of the toxin, LD_{50} data may provide insight into the ability of the various toxins to interact with PP-1.

Three key points arise from these studies. First, linearization of the peptide abolishes the ability of the peptide to exert toxic effects. Therefore, the peptide must be cyclic to bind to the phosphatase. Secondly, modifications of the Adda sidechain (see Fig. 1.15) have demonstrated that the overall shape of this unique amino acid is critical to microcystin interaction. Slight modifications, including demethylation of the C9 of the Adda sidechain, have little effect on microcystin toxicity. However, geometrical isomers of microcystins in which the C6 diene is changed from an extended *trans* E configuration to the *cis* Z configuration possess drastically reduced toxicity. These decreases in toxicity are reflected in 100-fold increases in the IC_{50} of the toxins toward phosphatases [6]. Finally, it has been demonstrated that the free carboxylic acid on the Glu residue of microcystins is important to toxicity. Any modification of this moiety results in complete loss of toxicity.

Studies with structural isomers of microcystins also demonstrate which parts of the toxin are not important to toxin-phosphatase interactions. For example, several isomers of microcystin-LR have a different amino acid in place of the variable arginine, including alanine, methionine, phenylalanine, leucine, and homoserine, with no appreciable change in toxicity. These results are also confirmed by inhibition of PP-1 by arginine-substituted microcystins [7], which all possess IC_{50} values comparable to microcystin-LR. Small effects are seen with microcystins in which the variable leucine is changed to another amino acid. When that amino acid is another hydrophobic residue, insignificant changes occur. Only slight increases in LD_{50} are observed when a charged residue, such as arginine, or methionine sulfoxide (Met(O)) is introduced in the leucine position [5].

Given that far fewer variants of the smaller cyclic pentapeptide nodularins (see Fig. 1.16) are known, it follows that less data is available concerning structural interactions of this inhibitor with PP-1. However, it has been shown that methylation of the carboxylic acid moiety of nodularin's glutamic acid sidechain nullifies its toxic effects, just as the same modification on microcystin abrogates its toxicity [5]. In addition, the 6(Z) Adda geometric isomer of nodularin is also ineffective as a toxin, reflecting the case with microcystins. This evidence suggests that microcystins and nodularins interact with PP-1 at the same site and reinforces the importance of the Adda sidechain and carboxylic acid moiety of the cyclic peptide glutamic acid residues to interactions with PP-1.

Insights Derived from Sequence Comparisons of PP-1 and PP-2A

Prior to the determination of the crystal structure of PP-1, comparisons of the inhibition of PP-1 and PP-2A by the marine toxins provided the most insight into the interaction of the phosphatases with the toxins. In this regard it is important to remember that three of the four major protein serine/threonine phosphatases are all members of the same gene family, sharing significant primary sequence identity (see Fig 1.4). PP-2A shares 49% identity with PP-1 in its catalytic core while PP-2B, also known as calcineurin, shares 40% identity with PP-1 in its catalytic domain (Table 5.1). Despite sharing such similarity, each phosphatase responds differently to the marine toxins. As noted earlier, PP-1 and PP-2A are inhibited equally by the cyclic peptide inhibitors, while PP-2A is 100-fold more sensitive to okadaic acid than PP-1. Conversely, PP-2B is relatively resistant to the marine toxins (see Table 1.2).

Table 5.1 Sequence alignment of the PPP family of protein serine/threonine phosphatases. The primary sequences of the catalytic domains of human PP-1α, human PP-2A [8], and bovine PP-2B [9] are shown. The extreme C-terminal region of PP-2B is not shown. Residues involved in metal binding in the active site are indicated by an asterisk (*), while those involved in substrate phosphate coordination are indicated by (#). The catalytic His-125 is in bold type. Residues involved in the PP-1c-binding domain (discussed in section 1.4) are indicated by (§). The L7 loops of the phosphatases are underlined.

```
PP-1α    1  MSDSEKLNLDSIIGRLLEVQGSRPGKNVQLTENEIRGLCLKSREIFLSQPILLELEAPLKI
PP-2A    1         MDEKVFTKELDQWIEQLNECKQLSESQVKSLCEKAKEILTKESNVQEVRCPVTV
PP-2B   27  HRLTAKEVFDNDGKPRVDILKAHLMKEGRLEETVALRIITEGASILRQEKNLLDIDAPVTV

PP-1α   62  CGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYVDRGKQSLETICLLLAYKIKYPENFFLLR
PP-2A   55  CGDCHGQFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLLVALKVR--ENITILR
PP-2B   88  CGDIHGQFFDLMKLFEVGGSPANTRYLFLGDYVDRGYFSIECVLYLWALKILYPKTLFLLR
              * *                         *  ##

PP-1α  123  GNHECASINRIYGFYDECKRRY-NIKLWKTFTDCFNCLPIAAIVDEKIFCCHGGLSPDLQS
PP-2A  114  GNHESRQITQVYGFYDECLRKYGNANVWKYFTDLFDYLPLTALVDGQIFCLHGGLSPSIDT
PP-2B  149  GNHECRHLTEYFTFKQECKIKYSE-RVYDACMDAFDCLPLAALMNQQFLCVHGGLSPEINT
             *#                                         § §    *

PP-1α  183  MEQIRRIMRPTDVPDQGLLCDLLWSDPDKDVQG-------WGENDRGVSFTFGAEVVAKFL
PP-2A  175  LDHIRALDRLQEVPHEGPMCDLLWSDPD-DRGG-------WGISPRGAGYTFGQDISETFN
PP-2B  209  LDDIRKLDRFKEPPAYGPMCDILWSDPLEDFGNEKTQEHFTHNTVRGCSYFYSYPAVCEFL
                                                     #

PP-1α  237  HKHDLDLICRAHQVVEDGYEFFAKRQ------LVTLFSAPNYCGEFDNAGAMMSVDETLMC
PP-2A  228  HANGLTLVSRAHQLVMEGYNWCHDRN------VVTIFSAPNYCYRCGNQAAIMEIDDTLKY
PP-2B  270  QHNNLLSILRAHEAQDAGYRMYRKSQTTGFPSLITIFSAPNYLDVYNNKAAVLKYENNVMN
                 §§    *        §                    *              § § §

PP-1α  293  SFQILKPADKNKGKYGQFSGLNPGGRPITPPRNSAKAKK
PP-2A  289  SFLQFDPAPRRGEPHVTRRTPDYFL
PP-2B  331  IRQFNCSPHPYWLPNFMDVFTWSLPFVGEKVTEMLVNVLNICSDDELGSEEDGFDGATAAA
            §
```

An initial observation that some hamster cell lines were resistant to okadaic acid toxicity lead to the finding that a single point mutation in the gene encoding PP-2A was responsible for this effect. This mutation, which causes the substitution of Cys-269 of PP-2A with a glycine residue, caused PP-2A activity to be 2 to 4-times more resistant to okadaic acid in these cells [10]. It was noted that this cysteine is part of a C-terminal four amino acid-motif that is variable between PP-1 and PP-2A. This motif lies in a turn between β-strands 12 and 13 of the PP-1 structure, also known as the L7 loop of PP-2B

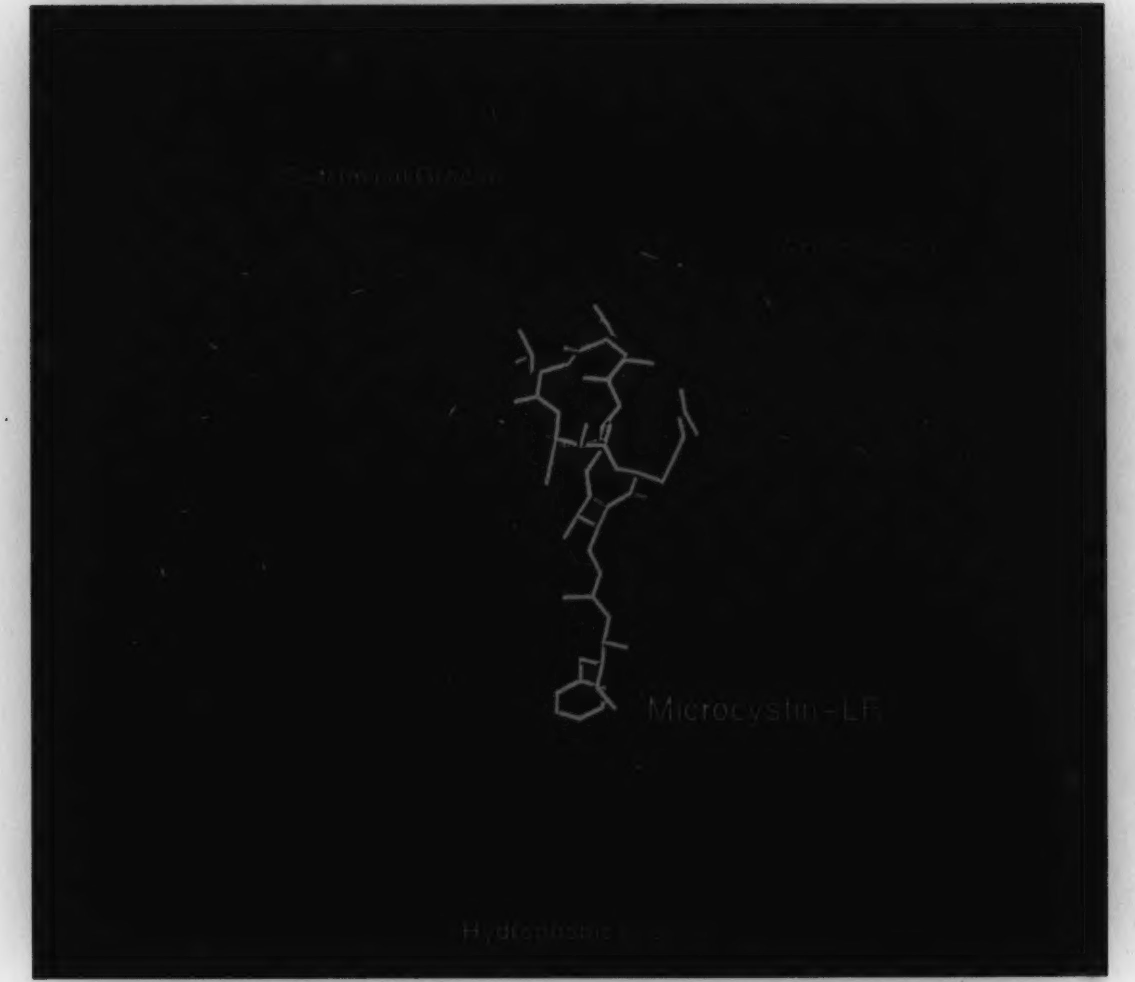
[9], which I will refer to as the L7 loop for all the phosphatases hereafter. The production of a chimeric form of PP-1 through mutagenesis of its L7 loop such that it possessed the amino acids present in the L7 loop of PP-2A resulted in a mutant form of PP-1 that was 10-times more sensitive to okadaic acid than the wild type enzyme [11]. Thus, it was concluded that the L7 loop of the phosphatases must form part of an okadaic acid-binding domain and that the different amino acid composition of PP-1 and PP-2A in this region accounts in part for the different sensitivity of these phosphatases to the dinoflagellate-derived toxin.

This work also shed light on the interaction of the peptide inhibitors with PP-1 and PP-2A. Since both microcystin and nodularin inhibit the binding of okadaic acid to cellular proteins [12,13], it was suggested that these toxins bind their target proteins on the same or overlapping sites, although it was initially difficult to believe that such chemically disparate compounds could interact at a common single site. Indeed, the chimeric form of PP-1 possessing the PP-2A L7 loop exhibited modest increases in resistance to the peptide inhibitors, strengthening the view that different toxin-binding sites that overlap slightly exist on PP-1 and PP-2A. However, the possibility that a common site might exist made the region surrounding the L7 loop of PP-1 and PP-2A of particular interest. Mutagenesis studies of the region surrounding the L7 loop of PP-1 revealed that Tyr-272 plays a crucial role in toxin binding [14]. Mutation of this residue caused significant increases in toxin resistance of the phosphatase. In addition, it has been determined that microcystins form a covalent linkage with PP-1 between the Mdha sidechain of the toxin and Cys-273 [15,16]. This covalent linkage further supports the hypothesis that the marine toxin-binding domain of PP-1 includes the L7 loop of the phosphatase, since Cys-273 joins Tyr-272 and the four amino acids identified as playing a role in okadaic acid sensitivity.

Insights Derived from the Crystal Structure of Microcystin-LR Bound to PP-1

With the advent of the crystal structure of PP-1 with microcystin-LR bound in 1995, precise structural interactions between the inhibitor and the phosphatase could be observed directly for the first time (Fig. 5.1)[17]. The catalytic site of PP-1 is located at the convergence of three surface grooves, forming a Y-shape. These three grooves have been termed the hydrophobic groove, a conserved arrangement of hydrophobic sidechains also found in PP-2A and PP-2B, the acidic groove, containing a number of acidic sidechains, and the C-terminal groove, which is formed in part by the C-terminus of the

Fig. 5.1 Crystal structure of microcystin-LR bound to PP-1 [17]. The active site of PP-1 is located at the confluence of three surface grooves forming a Y-shape in the orientation illustrated. The hydrophobic groove contains hydrophobic residues and is believed to be involved in substrate recognition. The acidic groove contains several acidic residues and is also believed to be involved in binding basic features of phosphatase substrates. The C-terminal groove contains structures that are part of the C-terminal of the phosphatase. Microcystin-LR is bound in the active site of PP-1 and interacts with residues that are important to the catalytic mechanism of the phosphatase, thereby completely blocking the active site. Most notably, the hydrophobic Adda sidechain of microcystin-LR interacts with the hydrophobic groove of PP-1. In addition, the Mdha group of microcystin-LR forms a covalent bond with Cys-273 of PP-1 which is in the L7 loop just above microcystin-LR in this orientation. PP-1 is in a similar orientation to Fig. 1.10. Hydrogen atoms of microcystin-LR are not shown for clarity. This figure was generated using Insight II software.



Microcystin-LR
Hydrophobic

protein. The amino acids that bind the two metal ions in the binuclear metal catalytic center (see Fig. 1.11) are found at the junction of these three grooves.

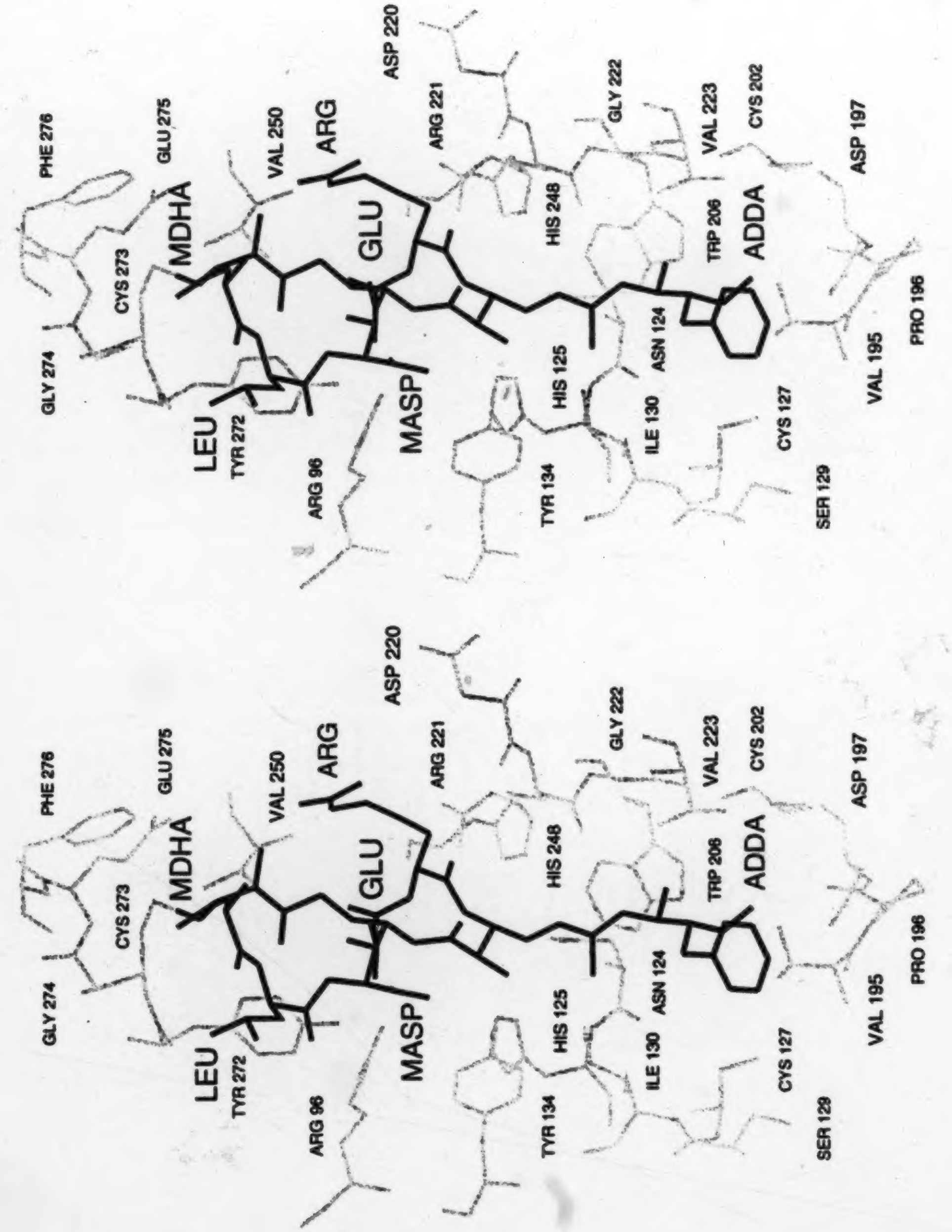
Microcystin-LR binds to PP-1 through interactions with three regions of the phosphatase (Fig. 5.2). Microcystin-LR coordinates with the two catalytic metal atoms indirectly via two water molecules through the carboxyl group of the glutamic acid and adjacent carbonyl oxygen of the toxin. This accounts for the observation that the carboxyl group of the microcystin glutamic acid is absolutely necessary for toxicity by the cyclic peptide inhibitor. In addition, the carboxyl group of the Masp of microcystin-LR interacts with Arg-96 and Tyr-134 of PP-1, thereby abrogating the involvement of Arg-96 in interaction with the phosphate of phosphatase substrates. Thus, microcystin-LR completely blocks access to the active center of the enzyme. This is in contrast to the well-known inhibitors of PP-2B, cyclosporin and FK506, which block access of protein substrates to the active site of PP-2B when bound to their cognate receptors, but do not disrupt access of the small non-physiological substrate *p*NPP, which is still dephosphorylated by PP-2B when cyclosporin or FK506 is bound [9,18].

Cyclic peptide toxin interactions with the C-terminal groove also occur at the L7 loop of PP-1. The crystal structure demonstrates that Tyr-272 packs closely to the leucine of microcystin-LR, suggesting that hydrophobic interactions may occur there. This explains the biochemical evidence that PP-1 sensitivity to microcystin-LR is decreased when Tyr-272 is mutated, especially when the aromatic moiety of Tyr-272 is replaced [14]. Similarly, as discussed previously, replacement of the leucine of microcystin with other hydrophobic residues has little effect on toxicity, whereas substitution with charged residues causes slight decreases in toxicity [4,5]. The covalent linkage between the Mdha sidechain of the toxin and Cys-273 of PP-1 was also observed in the crystal structure, verifying what was earlier shown employing other forms of biochemical analysis.

The third region of PP-1 with which microcystin-LR interacts is the hydrophobic groove, which accommodates the Adda sidechain of the toxin. The packing of the Adda group to the PP-1 hydrophobic groove is quite close, suggesting that a significant portion of the binding potential for the toxin is derived from hydrophobic interactions in this region. It is not surprising, therefore, that alterations in the absolute configuration of the Adda sidechain in the form of the 6(Z) geometric isomers lead to loss of inhibition of the toxin. This would completely alter the orientation of the Adda in the hydrophobic groove such that hydrophobic interactions are lost and thus microcystins do not bind.

In addition to explaining the biochemical evidence surrounding important interactions between microcystins and PP-1, the crystal structure also explains the

Fig. 5.2 Stereo view of the amino acids of PP-1 that lie within 4Å of bound microcystin-LR from the crystal structure of microcystin-LR bound to PP-1 [17]. The structure of microcystin-LR is shown in black, while the amino acids of PP-1 lying within 4Å are in grey. Larger labels refer to sidechains of microcystin-LR. According to the crystal structure, microcystin-LR makes extensive contacts with PP-1 throughout its active site, including several amino acid sidechains that are important for the catalytic mechanism of the phosphatase (see Fig. 1.11). These include the phosphate recognition amino acids Arg-96 and Arg-221, the catalytic nucleophile His-125, and metal binding residues His-248, Asn-124, and Tyr-272. The covalent bond that is formed between the Mdha group of microcystin-LR and Cys-273 of PP-1 is shown. Tyr-134 is believed to interact with the carboxyl group of the Masp sidechain of microcystin-LR and is thought to contribute to recognition of pSer or pThr of phosphatase substrates in the uninhibited enzyme. Trp-206 forms part of the hydrophobic groove of PP-1 which is believed to play an important role in the high affinity binding of the Adda sidechain of microcystin-LR. This view is in the same orientation as Fig. 5.1. Hydrogen atoms are not shown for clarity. This figure was generated using Insight II software.


PHE 276
GLU 275
GLY 274
CYS 273
MDHA
VAL 250
ARG
ASP 220
ARG 221
GLU
LEU
TYR 272
MASP
ARG 96
HIS 248
GLY 222
HIS 125
TYR 134
ILE 130
ASN 124
TRP 206
VAL 223
CYS 202
ADDA
ASP 197
CYS 127
SER 129
VAL 195
PRO 196

structural basis for features of microcystins that are unimportant for phosphatase binding. As discussed earlier, the variable amino acid arginine of microcystin-LR can be substituted with a variety of different amino acids with little effect on the inhibitory properties of the toxin. The crystal structure of microcystin bound to PP-1 demonstrates that the arginine sidechain of the toxin is directed away from the catalytic site of the phosphatase and is not involved in any productive interactions with the phosphatase. Therefore, it is logical that substitutions of this amino acid would not cause any change in inhibition characteristics of the toxin.

An additional insight into the interaction of microcystins and PP-1 derived from the crystal structures is that the conformation of microcystin-LR does not change significantly from its solution structure [19], which might contribute to the high affinity of PP-1 for the toxin. Comparison of the crystal structures of PP-1 with and without microcystin-LR bound indicates that the L7 loop of PP-1 shifts slightly to avoid steric conflict between Tyr-272 and the Mdha sidechain of microcystin-LR [20]. This disrupts the potential for interactions between the hydroxyl of Tyr-272 and one of the catalytic metal atoms in the active site via a water molecule (see Fig. 1.11), thereby destabilizing the catalytic conformation of the enzyme. Moreover, this observation suggests that flexibility of the L7 loop of PP-1 may be important to toxin binding and inhibition of the enzyme.

With the crystal structure data, the interactions between microcystin-LR and PP-1 are clearly seen. Previous workers have hypothesized that nodularin interacts with PP-1 in a similar fashion, since the solution structure of nodularin-V retains the important chemical and conformational structures of microcystins [19].

Insights Derived from Mutagenesis Studies Based on PP-1 Crystal Structures

The publication of the crystal structures of PP-1 raised new questions regarding the catalytic mechanism of PP-1 and interactions between this phosphatase and microcystin-LR. Recently, several groups have investigated the contribution of various sidechains of PP-1 to catalysis through mutagenesis of important sidechains [21,22]. These studies verified the importance of several proposed catalytic residues, including Arg-96 and Arg-221, which are involved in coordinating with the phosphate group of the substrate and stabilizing the pentacoordinate transition state, and Asn-124 and His-248, which coordinate one of the metal atoms in the catalytic site. In addition, mutations of His-125 lead to mutant PP-1 with no detectable activity, supporting the hypothesis that this residue may be the attacking nucleophile in the catalytic mechanism.

Some of the mutant forms of PP-1 designed to test the catalytic mechanism of PP-1 were also tested for their ability to be inhibited by microcystin-LR and okadaic acid. One caveat to these studies is that by altering amino acid sidechains involved in catalysis, mutant forms of PP-1 possess compromised catalytic efficiency. Thus, direct comparison with wild type enzyme is not possible. However, insight into the binding of the marine toxins is possible as long as the mutant PP-1 possesses some catalytic activity. In this case, the binding of the inhibitor is monitored in comparison to the activity of the mutant PP-1 in the absence of the inhibitor. In the studies I performed for this chapter, the same strategy had to be employed for some of the data I collected, as we shall see. The previous studies demonstrated that mutations in the acidic groove of PP-1 had no effect on marine toxin binding and inhibition, verifying the crystal structure data which suggest that microcystin-LR does not interact with the acidic groove.

In the case of microcystin-LR, mutation of the phosphate-coordinating Arg-96 or Arg-221 caused large increases in IC_{50} for the mutant PP-1; in the order of 1500-fold increases in resistance. These results verified the crystal structure data which suggest that Arg-96 interacts with the carboxyl group of the Masp sidechain. It is also possible that the carbonyl oxygen of the arginine sidechain of microcystin might interact with Arg-221, explaining that result.

Large undetermined increases in the IC_{50} of okadaic acid were also observed for mutations of the two arginine residues. These increases in toxin resistance were not quantified because of the large concentrations of okadaic acid required to test the mutants. As discussed earlier, PP-1 is 100-fold less sensitive to okadaic acid than microcystin-LR and thus the same amount of inhibition requires 100 times more okadaic acid. As a result, dose response above 5 µM okadaic acid was not determined in these studies. Therefore, it appears that the two phosphate-coordinating arginine residues are also important for okadaic acid interaction with PP-1.

Significant increases in marine toxin resistance were observed for mutations of Asn-124 and His-248. These amino acids are located at the junction of the hydrophobic groove of PP-1 with the catalytic site, suggesting that modification of these positions might disrupt the binding of the Adda sidechain of microcystins, which we have seen is an important feature for toxin sensitivity. Similar increases in resistance were seen for these mutants when okadaic acid was employed as the inhibitor.

Taken together, all of the data presented to this point suggest that the marine toxins interact with several active site residues, in addition to the L7 loop. Moreover, the evidence suggests that okadaic acid binds to the same site as microcystin-LR. Despite their dissimilar linear chemical structures, early molecular modeling studies indicated that the cyclic peptide inhibitors possess a similar overall shape, with the carboxyl group of okadaic acid occupying a position similar to that of the carboxyl group of the Glu of microcystin and nodularin [23]. This hypothesis is supported by biochemical evidence that demonstrates that modification of either the carboxyl group of okadaic acid or the carboxyl group of microcystin-LR abrogates inhibition of phosphatase activity by the marine toxin.

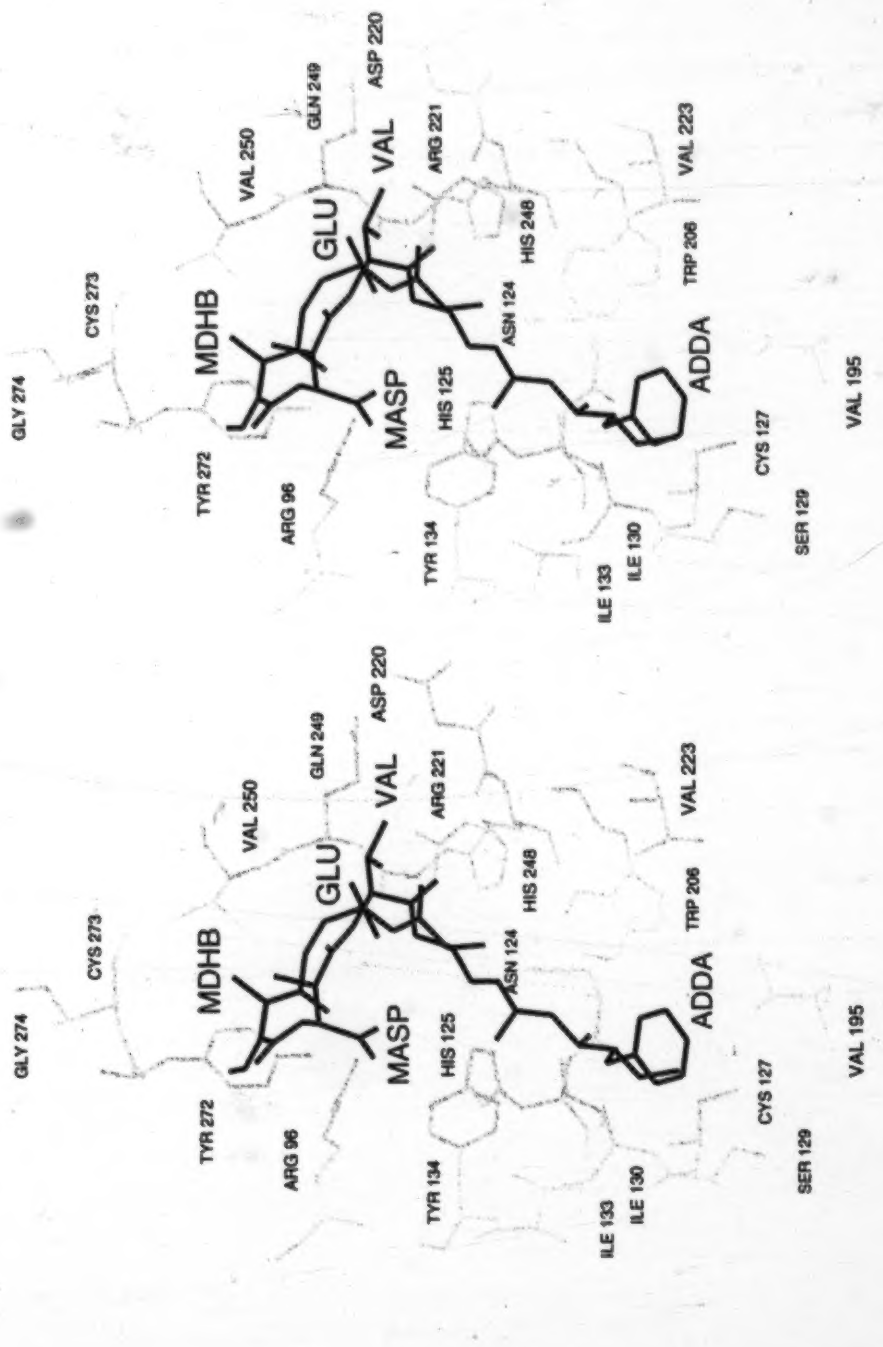
Molecular Modeling of Nodularin Bound to PP-1

Determination of the crystal structure of microcystin-LR bound to PP-1 was a great advance in our understanding of the molecular mechanisms of inhibition of PP-1 by the microcystins. Three-dimensional free structures for nodularin [19] and okadaic acid are also known. It has been noted that the structure of microcystin-LR does not change significantly upon binding to PP-1 [24], suggesting that this might contribute to the high affinity of microcystin-LR for PP-1. Since the free solution structures of microcystin-LR and nodularin-V are similar [19], it was hypothesized that the structure of nodularin-V also does not change significantly upon binding to the phosphatase. Therefore, the free solution structure of nodularin-V has been docked onto the crystal structure of PP-1, providing a model of the molecular mechanism of inhibition of PP-1 by this small cyclic peptide inhibitor (Fig. 5.3).

The model of nodularin-V bound to PP-1 predicts that similar PP-1 amino acid residues lie within 4Å of the toxin with respect to the microcystin-LR bound structure, including those important interactions between the Adda sidechain and the Glu carboxyl group. It was noted that, due to the small cyclic ring of the pentapeptide relative to the larger microcystin heptapeptide, the structure of nodularin is farther from some possible interactions with the L7 loop of PP-1. However, a significant interaction between the Mdhb group of nodularin, which assumes a position similar to the leucine sidechain of microcystin-LR in the docked model, and Tyr-272 of PP-1 is predicted to occur. This supports the hypothesis that interactions between Tyr-272 and the marine toxins are important to their inhibition of the phosphatase. Moreover, since PP-1 is equally sensitive to both nodularin and microcystin-LR, the model suggests that interactions with the later amino acids of the L7 loop (amino acids 274-277), which may not occur with nodularin, may not be important for the inhibition of PP-1 by both cyclic peptides. This contrasts the case of okadaic acid for which the L7 loop has been shown to be an important determinant of its toxicity [11].

In addition, the molecular model of nodularin-V bound to the crystal structure of PP-1 provides a mechanism for the lack of a covalent linkage between it and PP-1, while

Fig 5.3 Stereo view of the amino acids of PP-1 that lie within 4Å of the docked free solution structure of nodularin-V employing molecular modeling [24]. The structure of nodularin-V is shown in black, while the amino acids of PP-1 that lie within 4Å of this structure are in grey. Larger labels correspond to sidechains of nodularin-V. The orientation of PP-1 is exactly the same as that depicted in Fig. 5.2. The contacts predicted by this model are very similar to those made by microcystin-LR as observed in the crystal structure (see Fig. 5.2). Being a smaller compound, however, nodularin-V lacks extensive contact with the L7 loop of PP-1 and some contacts mediated by the end of its Adda sidechain. Due to the slightly different orientation of the Adda sidechain of nodularin-V relative to that of microcystin-LR, an additional contact with Ile-133 of PP-1 is observed. Hydrogen atoms are not shown for clarity. This figure was generated using Insight II software.


GLY 274
CYS 273
TYR 272
ARG 96
MDHB
GLU
VAL
VAL 250
GLN 249
ASP 220
ARG 221
HIS 248
ASN 124
MASP
HIS 125
TYR 134
ADDA
TRP 206
VAL 223
ILE 133
ILE 130
CYS 127
SER 129
VAL 195

microcystin-LR forms a covalent link. According to the model, the comparable reactive Mdhb group of nodularin assumes a radically different orientation with respect to Cys-273 of PP-1 that forms the covalent bond with microcystin-LR. As noted above, the Mdhb group of nodularin assumes a position similar to that of the leucine sidechain of microcystin-LR. In such a position, the Mdhb group is 10Å from Cys-273 and is therefore too far away to productively react. Indeed, the Mdhb group of nodularin is predicted to be directed away from the phosphatase and be accessible to solvent, where the reactive Mdha group of microcystin-LR is directed inward and reacts with Cys-273. It has been hypothesized that the predicted exposure of the reactive Mdhb group of nodularin to solvent may account for the unique tumour initiating properties of nodularin [24,25].

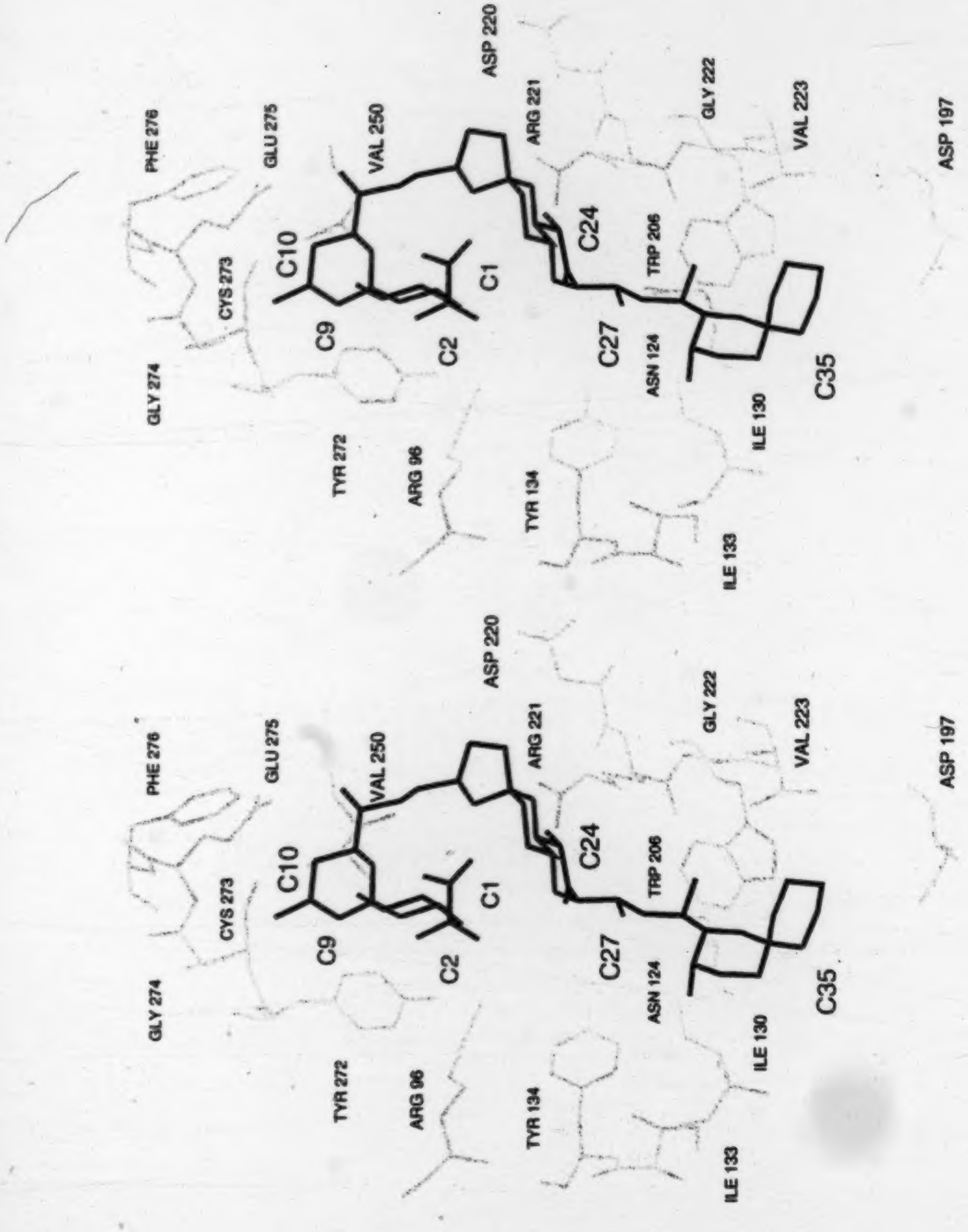
Molecular Modeling of Okadaic Acid Bound to PP-1

Previously, it was noted that microcystin-LR and okadaic acid share some common structural features [23]. Comparison of the crystal structures of free okadaic acid and the bound form of microcystin-LR verified this initial hypothesis [24]. This study indicated that the hydrophobic end of okadaic acid (to the right in Fig. 1.14) overlaps well with the hydrophobic Adda sidechain of microcystin-LR. Similarly, the C1 carboxyl group of okadaic acid also overlaps well with the carboxyl group of the glutamic acid sidechain of microcystin-LR. Based on the assumption that the free structures of the toxins do not change significantly upon binding, the free structure of okadaic acid was also docked onto the crystal structure of PP-1 and a model of the interaction of PP-1 with the polyether fatty acid inhibitor obtained (Fig. 5.4) [24].

This molecular model predicts that the hydrophobic region of okadaic acid fits into the hydrophobic groove occupied by the Adda sidechain of microcystin-LR. In addition, the C1 carboxyl group of okadaic acid appears to be near Arg-96 and Tyr-272 of PP-1, similar to the carboxyl group of glutamic acid and the adjacent carbonyl from Masp on microcystin-LR. Thus, the docked okadaic acid model is supported by biochemical evidence discussed above which demonstrated that modification of the C1 carboxyl group of okadaic acid results in drastic loss of phosphatase inhibition. Overall, the model suggests that okadaic acid and the cyclic peptide inhibitors bind to the same region of PP-1 employing many of the same interactions.

Together with the crystal structure of microcystin-LR bound to PP-1, the molecular models of nodularin and okadaic acid docked onto the crystal structure of PP-1 provide substantial insight into the molecular mechanism of inhibition of PP-1 by the marine toxins. The purpose of the work described in this chapter was to test the

Fig. 5.4 Stereo view of the amino acids of PP-1 that lie within 4Å of the docked free crystal structure of okadaic acid [24]. The structure of okadaic acid is shown in black, while the amino acids of PP-1 that lie within 4Å of it are in grey. The structure of okadaic acid is very similar to the free structure of microcystin-LR and fits in the same binding site in PP-1 as microcystin-LR. Larger labels refer to selected carbons of okadaic acid shown in Fig 1.14. The orientation of PP-1 is exactly the same as shown in Fig. 5.2. Despite dissimilar linear structures, the free structure of okadaic acid makes several of the same contacts with PP-1 as microcystin-LR does, although there is a lack of contacts with amino acids at the base of the hydrophobic groove, due to the shorter length of the hydrophobic tail of okadaic acid relative to the Adda sidechain of microcystin-LR. Hydrogen atoms are not shown for clarity. This figure was generated using Insight II software.


PHE 276
GLU 275
VAL 250
ASP 220
ARG 221
GLY 222
VAL 223
ASP 197
C10
CYS 273
GLY 274
C9
C1
C2
C24
TRP 206
C27
ASN 124
C35
TYR 272
ARG 96
TYR 134
ILE 130
ILE 133

interactions between the marine toxins and PP-1 predicted by the molecular models by mutating amino acid positions of PP-1 that may interact with the marine toxins and characterizing changes in marine toxin sensitivity of the mutants constructed. All of the mutants created were insoluble when expressed in *E. coli*. Therefore, the purification protocol developed in Chapter 4 was an invaluable tool, since most of the mutants would have otherwise been a tremendous challenge to purify prior to characterization.

In addition, I also compared the crystal structures of PP-1 and PP-2B and found some very interesting features, including a potential toxin binding site in PP-2B that appears to be blocked by specific structural features of PP-2B. Based on this comparison study, I formulated a hypothesis that explains the structural rationale for the relative resistance of PP-2B to the marine toxins despite sharing strikingly similar protein folding to PP-1 in the region surrounding the toxin binding site of that phosphatase. My hypothesis is the first to satisfactorily explain PP-2B marine toxin resistance. The interactions proposed by this hypothesis were also tested in the work outlined in this chapter by altering homologous amino acids in PP-1 and characterizing changes in marine toxin sensitivity. The work outlined in this chapter therefore provides significant advances in our understanding of the molecular mechanism of the resistance of PP-2B and the sensitivity of PP-1 to the marine toxins.

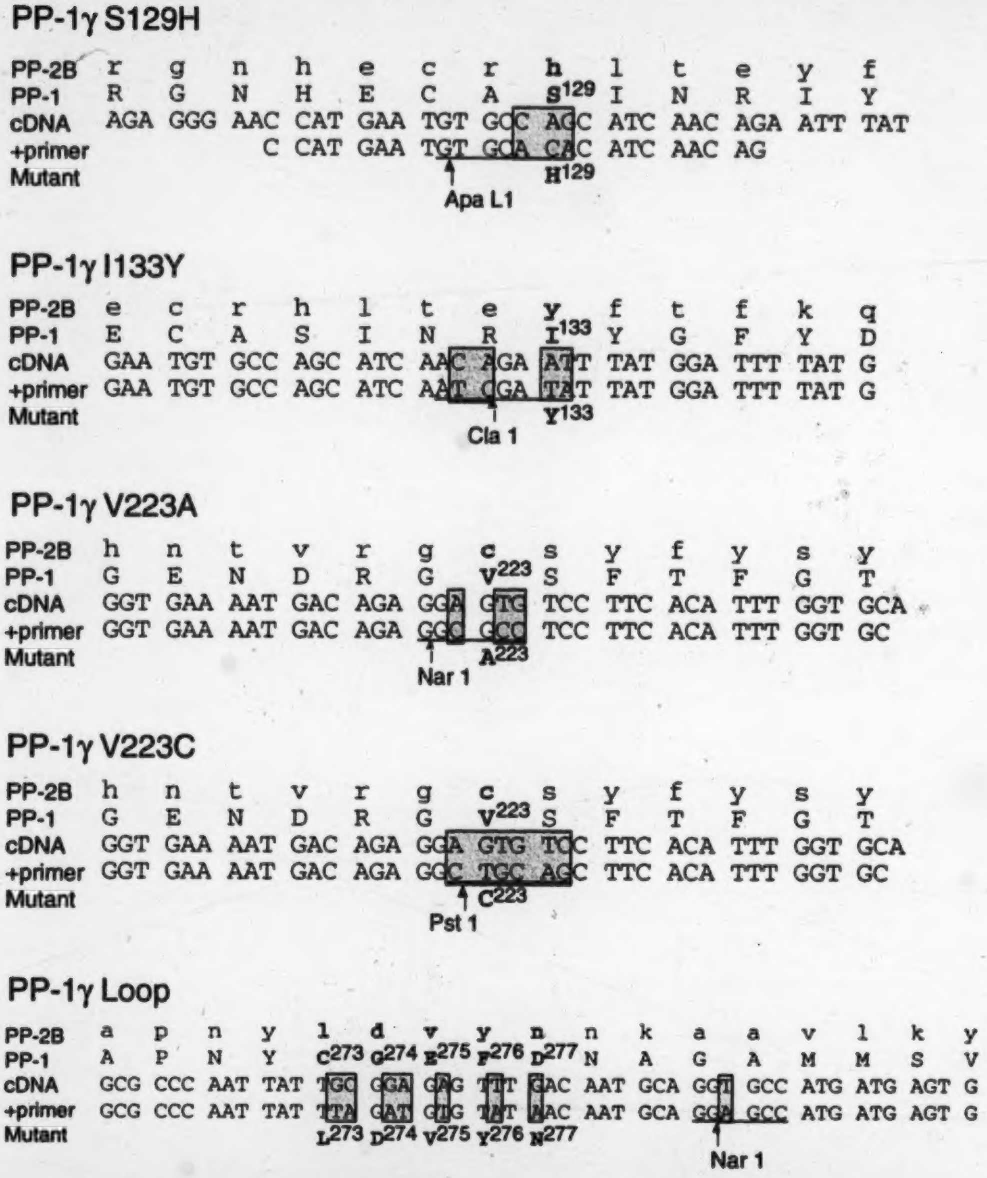
5.2 ■ MATERIALS AND METHODS

Molecular Modeling

The crystal structures of recombinant rat PP-1 [17] and bovine PP-2B [9] were superimposed using Insight II software, version 2.3 (Biosym Technologies). Metropolis Monte Carlo docking of nodularin-V and okadaic acid onto the crystal structure of PP-1 was achieved employing the Monte Carlo macro in Insight II software as performed by previous workers [24]. Docking of microcystin-LR onto the crystal structure of PP-2B was accomplished with the Monte Carlo macro of Insight II software.

Mutagenesis of PP-1γ

Human PP-1γ was isolated from a human teratocarcinoma cDNA library (Stratagene). The cDNA was inserted into the sequencing vector pKS$^-$ (Stratagene) employing NdeI and XbaI restriction sites. Mutant forms of PP-1γ were constructed using the Quikchange Mutagenesis System (Stratagene) employing the oligonucleotides outlined in Fig. 5.5 and their antisense counterparts (DNA Core Facility, University of Alberta). DNA sequencing was performed to verify the correctness of all the constructs produced. The



PP-1γ S129H

PP-2B	r	g	n	h	e	c	r	h	l	t	e	y	f
PP-1	R	G	N	H	E	C	A	S^{129}	I	N	R	I	Y
cDNA	AGA	GGG	AAC	CAT	GAA	TGT	GCC	AGC	ATC	AAC	AGA	ATT	TAT
+primer			C	CAT	GAA	TGT	GCA	CAC	ATC	AAC	AG		
Mutant								H^{129}					

PP-1γ I133Y

PP-2B	e	c	r	h	l	t	e	y	f	t	f	k	q
PP-1	E	C	A	S	I	N	R	I^{133}	Y	G	F	Y	D
cDNA	GAA	TGT	GCC	AGC	ATC	AAC	AGA	ATT	TAT	GGA	TTT	TAT	G
+primer	GAA	TGT	GCC	AGC	ATC	AAT	CGA	TAT	TAT	GGA	TTT	TAT	G
Mutant								Y^{133}					

PP-1γ V223A

PP-2B	h	n	t	v	r	g	c	s	y	f	y	s	y
PP-1	G	E	N	D	R	G	V^{223}	S	F	T	F	G	T
cDNA	GGT	GAA	AAT	GAC	AGA	GGA	GTG	TCC	TTC	ACA	TTT	GGT	GCA
+primer	GGT	GAA	AAT	GAC	AGA	GGC	GCC	TCC	TTC	ACA	TTT	GGT	GC
Mutant							A^{223}						

PP-1γ V223C

PP-2B	h	n	t	v	r	g	c	s	y	f	y	s	y
PP-1	G	E	N	D	R	G	V^{223}	S	F	T	F	G	T
cDNA	GGT	GAA	AAT	GAC	AGA	GGA	GTG	TCC	TTC	ACA	TTT	GGT	GCA
+primer	GGT	GAA	AAT	GAC	AGA	GGC	TGC	AGC	TTC	ACA	TTT	GGT	GC
Mutant							C^{223}						

PP-1γ Loop

PP-2B	a	p	n	y	l	d	v	y	n	n	k	a	a	v	l	k	y
PP-1	A	P	N	Y	C^{273}	G^{274}	E^{275}	F^{276}	D^{277}	N	A	G	A	M	M	S	V
cDNA	GCG	CCC	AAT	TAT	TGC	GGA	GAG	TTT	GAC	AAT	GCA	GGT	GCC	ATG	ATG	AGT	G
+primer	GCG	CCC	AAT	TAT	TTA	GAT	GTG	TAT	AAC	AAT	GCA	GGA	GCC	ATG	ATG	AGT	G
Mutant					L^{273}	D^{274}	V^{275}	Y^{276}	N^{277}								

Fig. 5.5 Mutagenesis strategies employed to produce various mutants of human PP-1γ as outlined in the Materials and Methods section. Shown are the single letter codes for the primary sequences of the homologous regions of PP-1 and PP-2B, followed by the coding region of the PP-1 cDNA for the PP-1 region. An oligonucleotide corresponding to the +primer was produced, along with its antisense counterpart, to produce the mutants of PP-1 employing the Stratagene Quikchange Mutagenesis System. Changes in the PP-1 cDNA are shaded. Silent mutations were also made to introduce novel restriction sites (underlined and labeled) to aid in the isolation of mutant DNA.

PP-1γ coding sequence contained in the NdeI-XbaI fragment of the resulting pKS$^-$ construct was then subcloned into the pCW expression vector for expression in *E. coli*. The PP-1γ–W206E mutant was constructed by Ms. H. A. Luu.

Purification of Recombinant Mutant PP-1γ

The mutant forms of PP-1γ expressed in *E. coli* exhibited variable solubility. Recombinant insoluble PP-1γ was purified from inclusion bodies employing the protocol outlined in Chapter 4 for the purification of insoluble sds21 from inclusion bodies. Wild type PP-1γ and PP-1γ-Y134F and Y134A mutants were purified by Ms. H. A. Luu from the cytosolic fraction of *E. coli* cells employing the purification procedure outlined by Barford and Keller [26]. Protein concentrations were determined employing the protocol of Bradford [27]. The typical yield of purified protein from a 100 mL culture was approximately 0.1 mg employing the insoluble PP-1 purification protocol.

Dose response Curves

The dose response curves of the various purified PP-1γ mutants were determined employing ^{32}P- labeled phosphorylase *a* as substrate, as described in the Materials and Methods section of Chapter 4, save that all assays were performed in the presence of 1 mM $MnCl_2$, a requirement for maximum recombinant enzyme activity. Purified PP-1γ stock solutions were diluted in Solution B and assayed employing phosphorylase *a* as substrate until a 10 μL aliquot of diluted enzyme solution caused 20% ^{32}P release in a 30 μL assay, as described in the Materials and Methods section of Chapter 4. This dilution of enzyme stock solution was then employed to produce dose response curves for microcystin-LR, nodularin, and okadaic acid. Nodularin was from Calbiochem while microcystin-LR and okadaic acid were purified from cyanobacteria by Dr. M. Craig and *Prorocentrum lima* by Ms. H. A. Luu, respectively.

To 9 μL Solution B and 1 μL toxin was added an aliquot of diluted enzyme solution (10 μL). Okadaic acid was solubilized in 10% methanol while microcystin-LR and nodularin were in water. Control assays for each marine toxin were performed in which no toxin was added. In this case, 1 μL of water was substituted for microcystin-LR and nodularin, while 1 μL of 10% methanol was added instead of okadaic acid. Toxin-enzyme solutions were incubated at 30 °C for 5 min prior to the addition of 10 μL of phosphorylase *a*, as outlined in the Materials and Methods of Chapter 4. Treatment of each assay was identical to that described in Chapter 4. Duplicate assays for each concentration of toxin were performed. Microcystin-LR and okadaic acid dose response curves for the Y134A mutant were obtained by Ms. H. A. Luu.

Technical Challenges:
Specific Activity and the Effect of Enzyme Concentration

The W206E mutant had a specific activity about 150 times lower than that of the wild type enzyme. In addition, both of the Tyr-134 mutants displayed lower specific activity than the wild type. As noted earlier, it has been hypothesized that both Trp-206 and Tyr-134 of PP-1 play a role in the recognition and binding of pSer and pThr in the active site. Since the aim of the work described here was to examine the effect of PP-1 mutations on the response of the enzyme to marine toxins, the K_M for phosphorylase of each mutant was not determined. However, changes in the specific activity of the enzyme, especially in the case of the W206E mutant, tend to support the hypothesis that Tyr-134 and Trp-206 are involved in substrate recognition. Several of the mutant forms of PP-1 purified from insoluble inclusion bodies possessed specific activities that were markedly higher than that of the wild type. In these cases, the amount of mutant enzyme required to achieve 20% release of ^{32}P label in the phosphorylase assay was less than that required for wild type PP-1.

Since our strategy aimed at exploring the molecular mechanism of marine toxin inhibition of PP-1 was based on generating dose response curves for carefully selected mutants of PP-1, it is important at this point to comment on the theoretical and technical challenges of producing these data. As noted above, enzyme concentration is a factor in the dose response curves of the marine toxins due to their tight-binding characteristics. The assay I employed is the standard used throughout the protein phosphatase scientific community because it is very specific for PP-1 and PP-2A and is very sensitive. The sensitivity of this assay allows us to detect very low concentrations of marine toxins, which we have done at the University of Alberta to monitor microcystin concentrations in sources of freshwater for drinking and kidney dialysis and okadaic acid concentrations in mussel samples. Since the assay is so sensitive, changes in enzyme concentration are readily detectable. For example, Fig. 5.6 illustrates two dose response curves for the same enzyme, one done properly employing a dilution of enzyme that yields 20% ^{32}P release, and another done in error employing 4 times the amount of enzyme resulting in 68% release of ^{32}P label. An apparent increase in the IC_{50} is observed when the higher enzyme concentration is employed in the assay, suggesting an increase in resistance to the toxin. Alternatively, lower concentrations of enzyme yield dose response curves that suggest an increase in the sensitivity of the enzyme to the toxins.

Ideally, all of the enzymes employed would possess the same specific activity such that the same amount of enzyme could be added to each assay, thereby overcoming any enzyme concentration effects, such as those illustrated in Fig. 5.6. However, the

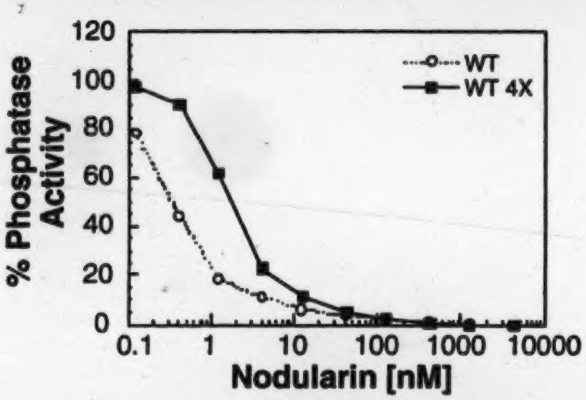


Fig. 5.6 The effect of enzyme concentration in the phosphorylase phosphatase assay employed to generate dose response curves for mutants of PP-1. Shown above are the dose response curves for nodularin of wild type PP-1 when the correct amount of enzyme was employed in the assay (open circles) to give 20% release of ^{32}P label in control assays without inhibitor. Conversely, when four times the concentration of wild type enzyme was employed (closed boxes), 68% release of ^{32}P label was measured in control assays in the absence of inhibitor. At the higher concentration of enzyme, the dose response curve was shifted to the right, suggesting an increase in resistance to the inhibitor, even though exactly the same enzyme was tested in both cases. Conversely, if lower concentrations of enzyme were employed in the assays, we would expect the dose response curves to shift to the left of the correct wild type curve. Therefore, enzyme concentration is an important factor in the determination of dose response curves employing this sensitive assay. However, this was not possible in the cases of mutants that possessed very different changes in specific activity, as discussed in the text.

specific activities of the mutants produced did not match that of the wild type enzyme. On one hand, I was restricted by the practical limitations of the assay I employed, which required that linearity be maintained by diluting enzyme stock solutions until 20% release of ^{32}P label from the isotopically labeled phosphorylase substrate was achieved. On the other hand, I faced the effects of employing different enzyme concentrations in the assay. I decided that it was more practical to employ 20% release of ^{32}P label for all the enzymes examined.

An alternative would be to maintain a constant enzyme concentration for both the wild type and mutant enzyme for each dose response curve. In this manner, the dose response curve of a mutant could be compared directly with the wild type dose response curve. This would be most informative in cases where the mutation only had slight effects on the sensitivity of the enzyme to the toxins. In the end, however, this was not practical because several of the mutants possessed such different specific activities. As a result, I would have to try to match enzyme concentrations that would keep the percentage release of ^{32}P label within the range of linearity for the assay. This could simply not be done for most of the mutants. If I attempted to maintain 20% ^{32}P release for one of the enzymes, the other enzyme would be either too concentrated or too dilute. If the enzyme concentration was too high, non-linear enzyme activity would result. If the activity was too low, the amount of background radiation, which was always measured as control assays in the absence of enzyme, became a significant factor. The most extreme example is the W206E mutant, which required a concentration of enzyme approximately 150 times that of the wild type to achieve 20% ^{32}P release. If I kept the wild type concentration at a level for 20% ^{32}P release, the same concentration of W206E mutant would exhibit no detectable activity in the assay.

I decided to employ enzyme concentrations which would achieve 20% release of ^{32}P label in the assays I performed to generate the dose response curves outlined in this chapter. In this way, one of the factors in the assay would be held constant. In cases where significant changes in enzyme sensitivity occur, the effect of different concentrations of enzyme was a minor factor in the resulting dose response curves.

5.3 ■ RESULTS

Comparison of the Crystal Structures of PP-1 and PP-2B

Despite sharing strikingly similar protein folding in their catalytic core regions (Fig. 5.7), PP-1 is sensitive to inhibition by marine toxins while PP-2B is not (see Table 1.2). All of the mutant forms of PP-1 reviewed thus far correspond to changes in catalytic sidechains of PP-1 that also influence the inhibition of PP-1 by marine toxins. Curiously, all of these catalytic sidechains are structurally conserved in PP-2B (see Table 5.1).

I formulated the hypothesis that differences between PP-1 and PP-2B in specific amino acids involved in toxin binding to PP-1 underlie PP-2B resistance to marine toxins. Therefore, molecular modeling was employed to identify residues in PP-2B that differ from PP-1 in the region surrounding the marine toxin binding domain of PP-1. Using the crystal structure of microcystin-LR bound to PP-1 as a starting point, the structure of microcystin-LR was docked onto the crystal structure of PP-2B. Amino acid residues of PP-2B that might interfere with marine toxin binding were then identified.

Since PP-1 and PP-2B share such similar catalytic core protein folding, I tested the contribution of the PP-2B amino acids identified in the molecular modeling to the resistance of PP-2B to the marine toxins by mutating the homologous residue of PP-1 to that of PP-2B. I hypothesized that PP-2B amino acids which might contribute to toxin resistance could be substituted for their counterparts in PP-1. The dose response curves of these mutant forms of PP-1 could then be determined. If a particular amino acid of PP-2B contributes to toxin resistance, then the PP-1 mutant that possesses that amino acid

Fig. 5.7 Superimposition of ribbon diagrams of the crystal structures of the catalytic subunits of PP-1 (red, [17]) and PP-2B (green, [9]). The orientation of PP-1 in this figure is the same as that shown in Fig. 5.1. The C-terminal of PP-1 is shown at the top of this view, while the C-terminal of PP-2B forms an α-helix seen at the left of the structures. This PP-2B helix contains the binding domains for calmodulin and the B-subunit of PP-2B and the autoinhibitory region (see Fig. 1.4). PP-2B shares 40% identity with PP-1 in its catalytic domain, reflected by the striking similarity of their three-dimensional architecture. Amino acids that are important for catalysis in PP-1 are conserved in the primary and tertiary structure of PP-2B.

Protein Phosphatase-2B

might exhibit increased toxin resistance, similar to PP-2B. In this manner, specific amino acid positions could be tested for their contribution not only to PP-2B toxin resistance, but to PP-1 toxin sensitivity.

Analysis of Molecular Models of Microcystin-LR Docked onto PP-2B

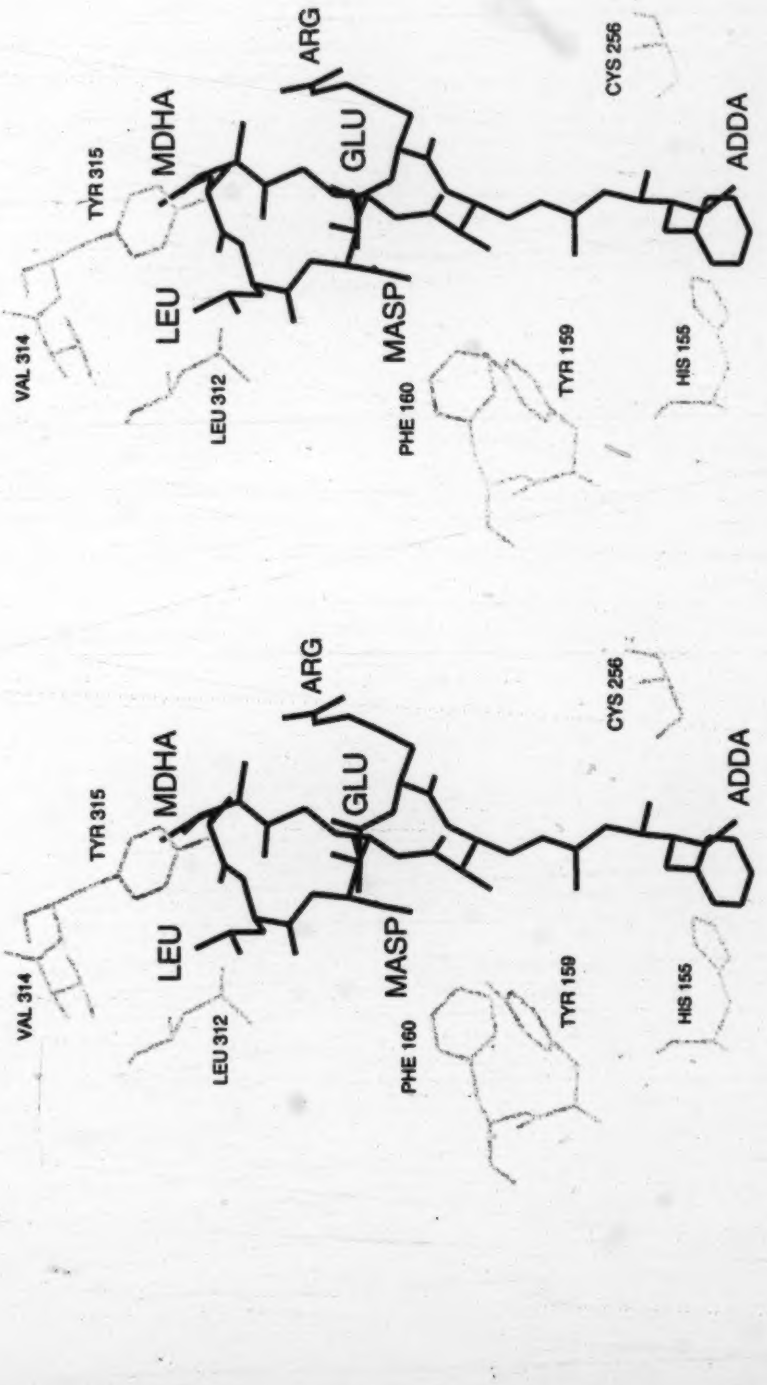
We employed molecular modeling to dock the structure of microcystin-LR onto PP-2B, employing the position of the toxin in complex with PP-1 as a reference point. We then identified PP-2B amino acids that lie within 4Å of the toxin. Twenty-two amino acids were identified in this manner, many of which comprise part of the conserved active site of the phosphatase. In fact, ten of the amino acids identified were conserved in PP-1. Three other amino acids represented conservative amino acid changes that did not cause significant structural differences between PP-1 and PP-2B. The interactions of the docked microcystin-LR structure with the nine remaining amino acids of PP-2B were examined in detail. Although the amino acids were different from PP-1, the interaction of the docked microcystin-LR structure with PP-2B was not considered significant for a further two amino acids. Finally, this process left us with seven amino acids that were candidates for mutagenesis of their comparable position in PP-1. The position of these seven amino acids of PP-2B relative to the docked microcystin-LR structure is shown in Fig. 5.8. A list of the comparable position in PP-1 is shown in Table 5.2.

Table 5.2 Amino acids identified through molecular modeling as lying within 4Å of microcystin-LR docked onto the crystal structure of PP-2B and possessing a structurally dissimilar amino acid in PP-1 in the homologous position. These amino acids became candidates for our mutagenesis strategy that involved substituting the PP-2B residue into PP-1 and then measuring increases in toxin resistance of the PP-1 mutant.

PP-1	PP-2B
Ser-129	His-155
Ile-133	Tyr-159
Tyr-134	Phe-160
Val-223	Cys-256
Cys-273	Leu-312
Glu-275	Val-314
Phe-276	Tyr-315

In addition to the amino acids identified through the docking of microcystin-LR onto the crystal structure of PP-2B, other residues of PP-1 were marked for mutagenesis based on the models of the free structures of the marine toxins docked onto the crystal structure of PP-1 (see Figs. 5.3 and Fig. 5.4). Two main candidates were given highest

Fig. 5.8 Amino acids of PP-2B that lie within 4Å of the docked bound structure of microcystin-LR which might block marine toxin binding. The structure of microcystin-LR is shown in black, while the amino acids of PP-2B are in grey. The orientation of microcystin-LR is exactly the same as that shown in Fig. 5.2. The amino acids depicted here were substituted into PP-1 and the dose response curves of the resulting PP-1 mutants measured. If an amino acid shown here contributes to the relative resistance of PP-2B to the marine toxins, we would expect to observe an increase in the resistance of the PP-1 mutant that harbours that amino acid. Hydrogen atoms are not shown for clarity. This figure was generated using Insight II software.


ARG
CYS 256
MDHA
GLU
ADDA
TYR 315
VAL 314
LEU
LEU 312
MASP
PHE 160
TYR 159
HIS 155
ARG
CYS 256
MDHA
GLU
ADDA
TYR 315
VAL 314
LEU
LEU 312
MASP
PHE 160
TYR 159
HIS 155

priority. These were PP-1 residues Trp-206 and Tyr-134. The sidechains of these two residues are positioned on either side of the hydrophobic groove of PP-1 and are hypothesized to interact with the pSer or pThr of the substrate [28]. In addition, Tyr-134 is also thought to interact with the carboxyl group of the Masp residue of microcystin-LR through hydrogen bonding [17]. Remarkably, the comparable amino acid to Tyr-134 of PP-1 in PP-2B (Phe-160) was identified in our molecular modeling strategy described above. We were therefore excited about the possibility that a change in amino acids in this position in PP-2B may contribute to the resistance of PP-2B and the sensitivity of PP-1 to the marine toxins.

In PP-2B, Trp-206 of PP-1 is conserved as Trp-232. This residue was among the amino acids in our initial identification of residues lying within 4Å of the PP-2B-docked microcystin-LR. Models of the toxins bound to PP-1 reveal that Trp-206 is a key amino acid in the hydrophobic groove of PP-1 which is occupied by the Adda sidechain of microcystin-LR and nodularin and the hydrophobic tail of okadaic acid. Since hydrophobic interactions are important for the binding of toxins to PP-1, it was of interest to examine the effect of mutating this amino acid to a charged glutamic acid residue.

Purification of Insoluble Mutant PP-1γ from *E. coli*

The experimental strategy I employed to analyze the molecular mechanism of the inhibition of PP-1 by marine toxins was to purify recombinant mutant forms of human PP-1γ and then produce dose response curves of the purified enzymes against microcystin-LR, nodularin, and okadaic acid. In this manner, amino acid positions could be identified that contribute to the sensitivity of PP-1 to the marine toxins.

All of the mutant forms of human PP-1γ constructed were insoluble to some degree when expressed in *E. coli* . The relative insolubility of a given mutation can be seen in Fig. 5.9 where the soluble and insoluble fractions of PP-1-expressing *E. coli* are compared. By inspection, we can see that the W206E mutant was almost completely insoluble, while the L7 Loop mutant was mostly soluble. Since the purification procedure I developed for insoluble PP-1 is very convenient, allowing purification of PP-1 in four days from starter culture to purified protein, I elected to employ this procedure for the purification of PP-1 mutants.

Mutations of the coding sequence of human PP-1γ were made as outlined in the Materials and Methods section and phosphatase expressed in DH-5α *E. coli* cells, save the V223C mutant, which was expressed in BL-21 *E. coli* cells. No apparent difference between expression of PP-1 in DH-5α or BL-21 cells was observed. A representative

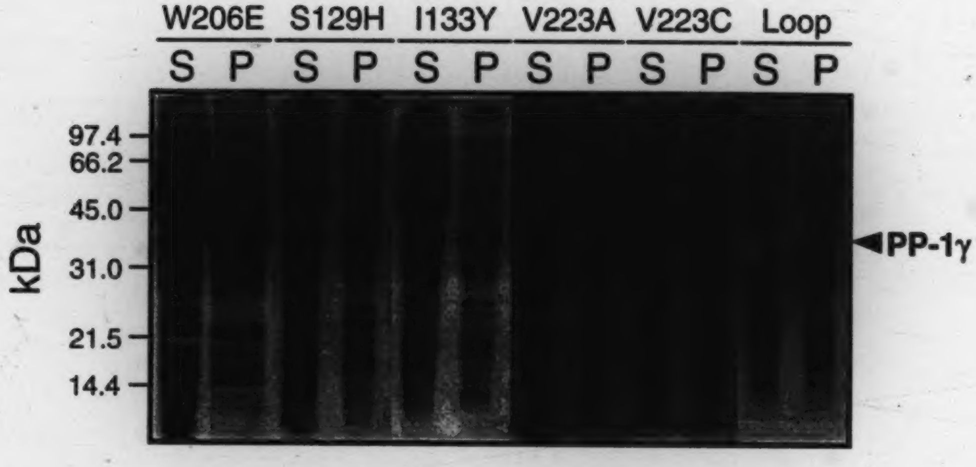

W206E
S129H
I133Y
V223A
V223C
Loop
S P S P S P S P S P S P
97.4
66.2
45.0
31.0
21.5
14.4
kDa
PP-1γ

Fig. 5.9 Solubility of various mutants of recombinant human PP-1γ overexpressed in *E. coli*. Cell lysates of overexpressing *E. coli* were centrifuged and the resulting supernatant (S) and pellet (P) analyzed by SDS-PAGE (12%). Visualization of the proteins with Coomassie Blue revealed the presence of insoluble PP-1 in the pellet fraction to varying degrees depending on the mutation, shown across the top.

example of the expression of a mutant form of human PP-1γ is shown in Fig. 5.10. The expression of PP-1γ in *E. coli* caused no significant changes in cellular growth.

Human PP-1γ was purified from inclusion bodies as described in the Materials and Methods section. A representative example of the progress of purification of a mutant of human PP-1γ expressed in *E. coli* is shown in Fig. 5.11. Figures illustrating data for the expression and purification of the other human PP-1γ mutants are shown in Appendix B. The specific activities of the purified PP-1 mutants are listed in Table 5.3.

Table 5.3 Specific activities of mutant PP-1. The concentration of the stock enzyme solution is also shown. Wild type, Y134F, and Y134A PP-1 were purified from the soluble fraction of *E. coli* cell lysates by Ms. H. A. Luu.

Mutant	Protein [μg/μL]	Sp. Act. [U/mg]
wild type	0.54	8.81
W206E	0.27	0.06
Y134F	0.74	3.35
Y134A	0.23	1.3
S129H	0.27	23.7
I133Y	0.20	46.0
V223A	0.34	10.6
V223C	0.35	13.1
L7 Loop	0.11	24.0

Characterization of the PP-1γ W206E Mutant

Trp-206 has been identified as lying within 4Å of bound microcystin-LR, nodularin, and okadaic acid in the crystal structure and molecular modeling studies. It is positioned close to the hydrophobic regions of the marine toxins when bound to PP-1, suggesting that it is involved in hydrophobic interactions that are important for toxin inhibition of the enzyme, as discussed above. We therefore mutated this residue to a charged glutamic acid to disrupt normal hydrophobic interactions in this region of the enzyme. Strikingly, this W206E mutant was completely insoluble when expressed in *E. coli*, and the insoluble PP-1 purification protocol that I had developed for sds21 was employed to purify the protein. Its specific activity was very low, supporting the hypothesis that Trp-206 is involved in substrate recognition.

The dose response curves for the W206E mutant are presented in Fig. 5.12. None of the toxins employed were able to completely inhibit the mutant phosphatase, even at

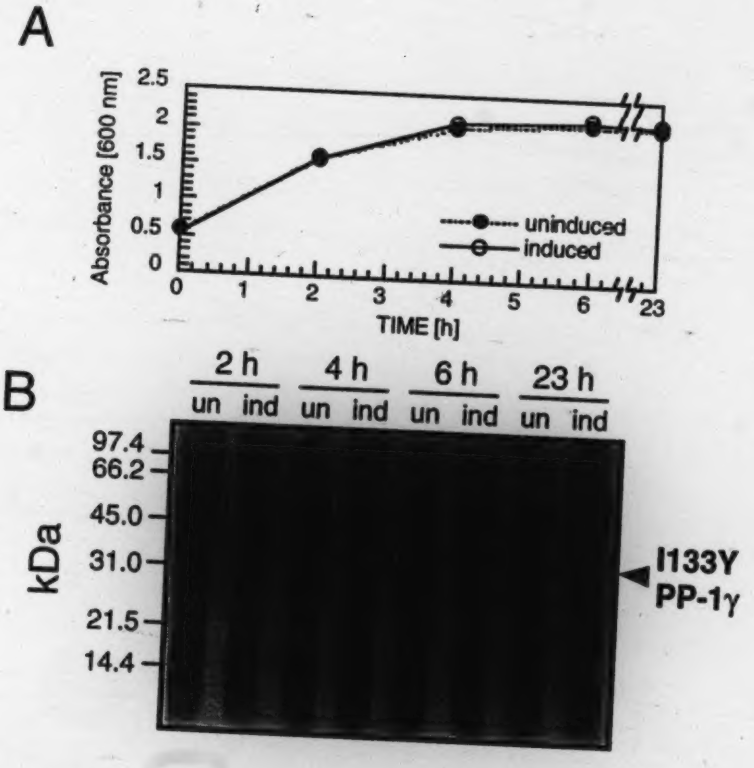


Fig. 5.10 Expression of the I133Y PP-1 mutant in *E. coli*. **A**. Cell density was monitored as a function of the absorbance of a culture at 600 nm. Expression of PP-1 in induced cultures had little effect on cellular growth. **B**. Induction of PP-1 expression in *E. coli* over time. Samples of parallel *E. coli* cultures, one uninduced (un) and one induced (ind), were taken at time points indicated across the top and whole cell lysates (10 μL) analyzed by SDS-PAGE (12%). Visualization of the proteins by Coomassie Blue staining revealed increasing amounts of I133Y PP-1 mutant in cells that had been induced.

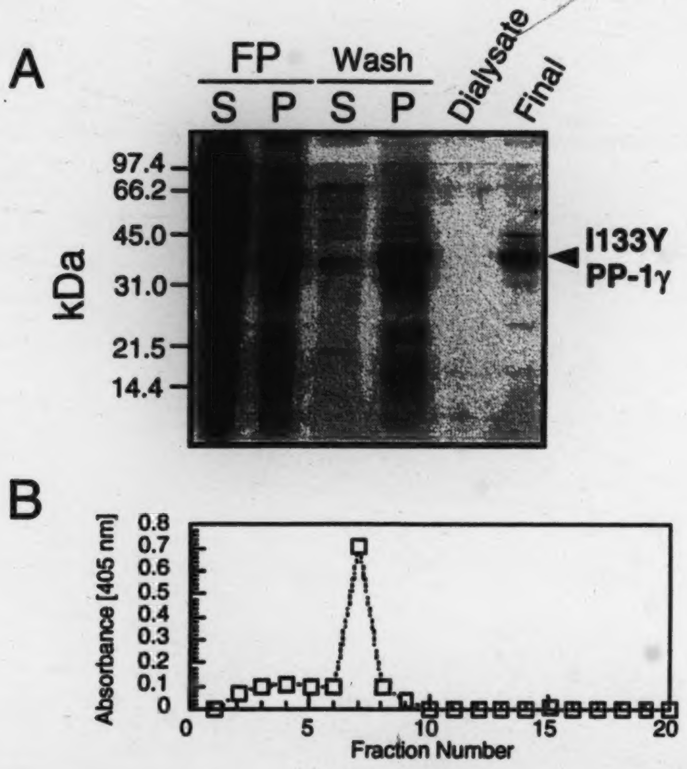


Fig. 5.11 Purification of I133Y PP-1 from insoluble inclusion bodies in *E. coli* employing the purification protocol outlined in Chapter 4 for the purification of sds21 from *E. coli*. **A.** Induced *E. coli* cell lysates produced employing a french press (FP) were centrifuged and the resulting pellet (P) separated from the supernatant (S). I133Y PP-1 is present in the insoluble pellet fraction. This pellet was then washed (Wash) in a Triton X-100 containing buffer and the supernatant (S) separated from the pellet (P). This pellet was solubilized and renatured and the resulting renaturation reaction dialyzed to remove salt. The dialysate (Dialysate) was loaded onto Heparin-Sepharose and I133Y PP-1 eluted. Active fractions were pooled and dialyzed against storage buffer to yield the final protein preparation (Final). The volume of each sample was 10 μL, except the dialysate for which 50 μL was analyzed **B.** Detection of I133Y PP-1 activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing *p*NPP as substrate. Phosphatase activity is measured as a function of the increase in absorbance of a reaction at 405 nm, as described in the Materials and Methods section of Chapter 3. The first five fractions were run in buffer containing 200 mM salt. The salt concentration was then increased to 450 mM for the next ten fractions and finally to 600 mM for the final five fractions. I133Y PP-1 eluted in the lead fractions containing 450 mM salt.

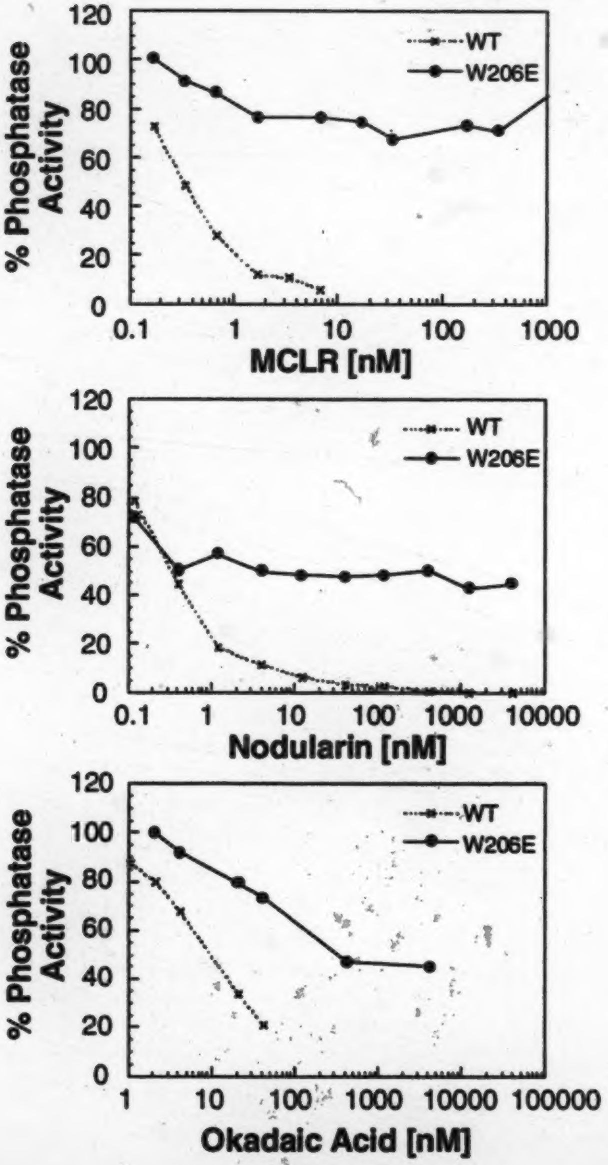


Fig. 5.12 Dose response curves of the W206E PP-1 mutant for the marine toxins. **A.** The dose response curve of the W206E PP-1 mutant for microcystin-LR relative to wild type enzyme. **B.** The dose response curve of the W206E PP-1 mutant for nodularin relative to wild type enzyme. **C.** The dose response curve of the W206E PP-1 mutant for okadaic acid relative to wild type enzyme. Each point represents the average of duplicate trials. The microcystin-LR curve was repeated three times with similar results. The okadaic acid curve was repeated twice with similar results.

extremely high doses. This was a very unusual result, since all dose response curves measured for PP-1 mutants documented thus far have demonstrated phosphatase activity that was either completely unaffected by the toxin or completely inhibited at concentrations of toxins equivalent to those I employed. This mutant, however, was neither completely inhibited nor completely unaffected. One could argue that the high concentration of enzyme required to obtain enough activity for the assay affected the dose response curve to yield such a curious result. However, other mutants that possess similarly compromised specific activities have been tested in the same assay as that used here, with resulting dose response curves that are typical [22]. Therefore, the curves obtained from the W206E mutant most likely are due to the mutation itself.

The W206A mutant has also been constructed and its dose response curves produced (Ms. H. A. Luu, pers. comm.). Like the W206E mutant, this form of PP-1 possesses very low specific activity and is quite insoluble. However, its dose response curves demonstrate that this mutant PP-1 is capable of being completely inhibited at extremely high concentrations of inhibitor. This suggests that the irregular dose response curves observed with the W206E mutant are the result of introducing a negatively charged residue into the hydrophobic groove of PP-1. The changes in IC_{50} for the W206A mutant are quite profound, with an approximately 20000-fold increase for microcystin-LR, 10000-fold increase for nodularin, and 5000-fold increase for okadaic acid. These results strongly support the hypothesis that hydrophobic interactions between PP-1 and the hydrophobic regions of the marine toxins play a critical role in the molecular mechanism of inhibition of PP-1 by the toxins.

Characterization of the PP-1γ Y134 Mutants

The crystal structures and modeled structures of the marine toxins bound to PP-1 suggested that Tyr-134 may be involved in binding the toxins. In the crystal structure, Tyr-134 may interact with the carboxyl group of the glutamic acid and Masp sidechains of microcystin-LR. Similarly, docked nodularin is hypothesized to interact with Tyr-134 through its glutamic acid and Masp sidechains. The structure of okadaic acid docked onto PP-1 suggests that Tyr-134 may interact with the carboxyl group of C1. In addition, our comparison of the structures of PP-1 and PP-2B suggested that the homologous Phe-160 of PP-2B may interact with marine toxins. It was important to determine if loss of a hydroxyl group in this position could contribute to PP-2B resistance to the marine toxins.

Two mutations of Tyr-134 were constructed and purified from the soluble fraction of *E. coli* cell lysates. One was the Y134F mutation in which the homologous phenylalanine residue present in PP-2B was substituted for Tyr-134. The second was the

Y134A mutant in which both the hydroxyl and the hydrophobic aromatic ring of the tyrosine residue were removed.

The dose response curves for both of the Tyr-134 mutants demonstrate that this residue is involved in toxin binding and inhibition of PP-1 (Fig. 5.13). With the Y134F mutant, it was observed that an approximately 10-fold increase in IC_{50} occurred for microcystin-LR and nodularin. Conversely, no significant change in IC_{50} was observed for the Y134F mutant when okadaic acid was present. These data suggest that the hydroxyl group of Tyr-134 is involved in a hydrogen bond-interaction with the cyclic peptide inhibitors, while interaction between okadaic acid and the hydroxyl of Tyr-134 does not seem to occur. Also, while the marine toxins appear to bind PP-1 in the same region, the precise interactions between the toxins and the phosphatase may be subtly different. These results also demonstrate the sensitivity of the assay employed, as these 10-fold increases in IC_{50} reflect the binding energy of a single hydrogen bond.

Removal of both the hydroxyl and aromatic ring of Tyr-134 in the Y134A mutant demonstrates the importance of hydrophobic interactions between the phosphatase and the inhibitors at this site. For microcystin-LR, a 600-fold increase in IC_{50} is observed. A smaller increase in resistance is noted for nodularin (approximately 100-fold increase in IC_{50}). Most strikingly, the IC_{50} of okadaic acid for the Y134A mutant increases about 1500 times relative to the wild type. These large increases suggest that hydrophobic interactions mediated by the presence of the aromatic ring of Tyr-134 in PP-1 significantly contribute to the binding and inhibition of PP-1 by the marine toxins. Moreover, these results strengthen the view that the marine toxins rely on the same interactions with the phosphatase to different degrees. In this case, hydrophobic interactions between Tyr-134 and okadaic acid are more important than the same interactions between the cyclic peptide inhibitors and PP-1.

Characterization of the PP-1γ S129H Mutant

Comparison of the PP-1 and PP-2B crystal structures revealed several amino acids in PP-2B that might contribute to its relative resistance to the marine toxins. One of these positions is His-155 of PP-2B, which appears to encroach upon the area that might be occupied by microcystin-LR. I therefore mutated the homologous Ser-129 of PP-1 to histidine and examined the ability of the resulting S129H mutant to be inhibited by the marine toxins.

The dose response curves of this mutant (Fig. 5.14) demonstrate no significant changes in inhibitor sensitivity. In purified form, the S129H mutant possessed higher specific activity that the wild type enzyme. Therefore, if we assume that the S129H

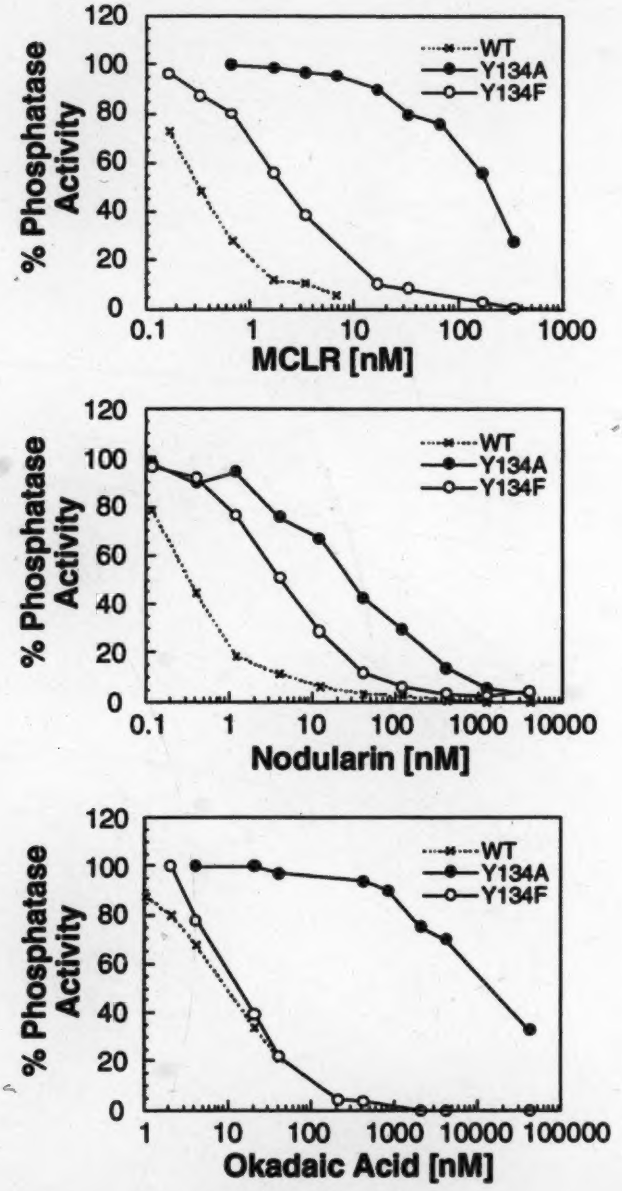


Fig. 5.13 Dose response curves of the Y134 PP-1 mutants for the marine toxins. A. The dose response curve of the Y134 PP-1 mutants for microcystin-LR relative to wild type enzyme. B. The dose response curve of the Y134 PP-1 mutants for nodularin relative to wild type enzyme. C. The dose response curve of the Y134 PP-1 mutants for okadaic acid relative to wild type enzyme. Each point represents the average of duplicate trials.

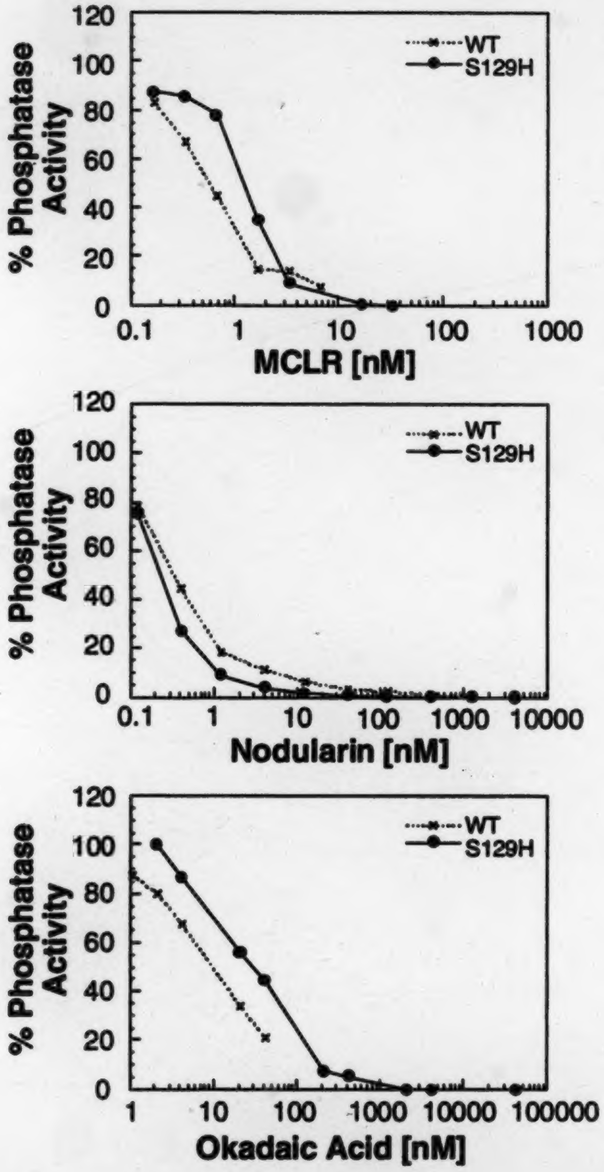


Fig. 5.14 Dose response curves of the S129H PP-1 mutant for the marine toxins. **A.** The dose response curve of the S129H PP-1 mutant for microcystin-LR relative to wild type enzyme. **B.** The dose response curve of the S129H PP-1 mutant for nodularin relative to wild type enzyme. **C.** The dose response curve of the S129H PP-1 mutant for okadaic acid relative to wild type enzyme. Each point represents the average of duplicate trials.

mutant possesses the same IC_{50} values as the wild type for the marine toxins, we might expect to see dose response curves that are shifted slightly to the left of the wild type curves, indicative of a lower concentration of enzyme in the assays. This is the case for nodularin, but does not occur for microcystin-LR and okadaic acid, where slight increases in toxin resistance are observed. While these changes are not as significant as those observed for the Y134A mutant, they might suggest that the presence of His-155 in PP-2B contributes slightly to increased resistance to the toxins. Conversely, these results might suggest that the presence of Ser-129 in PP-1 is conducive to inhibition by microcystin-LR and okadaic acid.

Characterization of the PP-1γ I133Y Mutant

In PP-2B, Tyr-159 projects into what might be the toxin binding region of this phosphatase. As such, Tyr-159 might act as a "doorway," blocking the entry of the toxins to the active site of the enzyme. The homologous amino acid in PP-1 is Ile-133, which is smaller and does not interfere with toxin binding according to the crystal structure data. The I133Y mutant was therefore generated and the dose response curves of it determined (Fig. 5.15).

The dose response curves for the I133Y mutant indicated no evidence of an increase in resistance to the cyclic peptide inhibitors. In fact, this mutant exhibited greater sensitivity toward the cyclic peptide inhibitors, although this may be attributed to enzyme concentration effects in the assay, as discussed in the Materials and Methods section. The dose response curves of the I133Y mutant for the cyclic peptide inhibitors likely represent no change in inhibitor sensitivity relative to the wild type. Thus, the increase in IC_{50} observed when okadaic acid is employed may be indicative of an increase in resistance of the PP-1 mutant of approximately 10-fold. Since the specific activity of the I133Y mutant is greater than wild type enzyme, precise quantitation of the increase in PP-1 resistance to okadaic acid for this mutant is not possible. However, these data suggest that the presence of Tyr-159 in PP-2B does not affect the potential of that phosphatase to bind the cyclic peptide inhibitors, while it may contribute to resistance to okadaic acid.

Characterization of the PP-1γ V223 Mutants

Cys-256 of PP-2B was identified as lying within 4Å of the docked structure of bound microcystin-LR. The homologous amino acid in PP-1 is Val-223. Two mutants of PP-1 at Val-223 were constructed to analyze its contribution to the sensitivity of the enzyme to marine toxins. These were the V223A and V223C mutants.

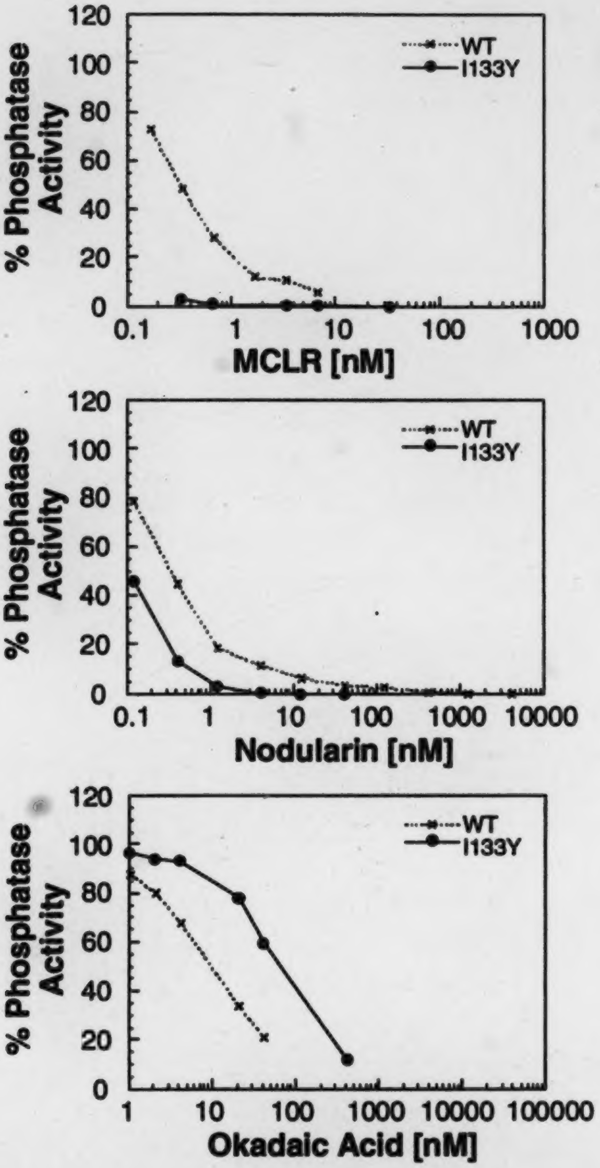


Fig. 5.15 Dose response curves of the I133Y PP-1 mutant for the marine toxins. **A.** The dose response curve of the I133Y PP-1 mutant for microcystin-LR relative to wild type enzyme. **B.** The dose response curve of the I133Y PP-1 mutant for nodularin relative to wild type enzyme. **C.** The dose response curve of the I133Y PP-1 mutant for okadaic acid relative to wild type enzyme. Each point represents the average of duplicate trials.

The dose response curves for the Val-223 mutants are depicted in Fig. 5.16. All of the dose response curves are shifted slightly to the left, indicative of the modest increase in specific activity of the mutants relative to the wild type. As such, it appears that Val-223 of PP-1 does not contribute to the marine toxin sensitivity of PP-1. Moreover, the presence of Cys-256 in PP-2B also does not appear to contribute to the resistance of that phosphatase to the marine toxins.

The L7 Loop of PP-2B is Radically Different from Its PP-1 Counterpart

All of the mutants described thus far in this chapter and in the literature [22] have not identified the molecular mechanism of marine toxin resistance of PP-2B. My data suggest that the PP-2B residues examined to this point do not contribute significantly to the striking resistance of PP-2B to the marine toxins. In addition, PP-2B possesses all of the active site residues mutated in previous studies [22] and in this study. Therefore, other structural differences between PP-1 and PP-2B most likely account for the resistance of PP-2B to the marine toxins.

The three remaining amino acids of PP-2B identified by our toxin docking strategy as lying with 4Å of microcystin-LR in the PP-2B crystal structure (see Table 5.2) are all within the L7 loop of PP-2B. Since it is already known that the L7 loop of PP-1 contributes to its higher resistance to okadaic acid over PP-2A [11], the possibility that the L7 loop of PP-2B might also be involved in toxin resistance in this phosphatase was particularly interesting.

We closely compared the crystal structures of PP-1 and PP-2B in the region surrounding the L7 loops of these phosphatases and observed a striking difference in their structure (Fig. 5.17). The conserved tyrosine residues, Tyr-272 of PP-1 and Tyr-311 of PP-2B are positioned in almost the same orientation in both phosphatases. This might be expected, especially when we consider that these tyrosine residues contribute to metal-binding in the similar active sites of these phosphatases. Also, two conserved asparagine residues, Asn-278 of PP-1 and Asn-317 of PP-2B, lie in very similar orientations. However, the interceding five amino acids, four of which were identified as contributing to the increased okadaic acid resistance of PP-1 relative to PP-2A [11], possess a radically different α-carbon backbone conformation. This results in the sidechains of PP-1 being directed away from microcystin-LR, thereby permitting the toxin to bind in the active site of PP-1. The L7 loop of PP-2B, however, is configured in such a way that its side chains, particularly those identified with our molecular modeling strategy, are expected to be directed into the toxin binding site.

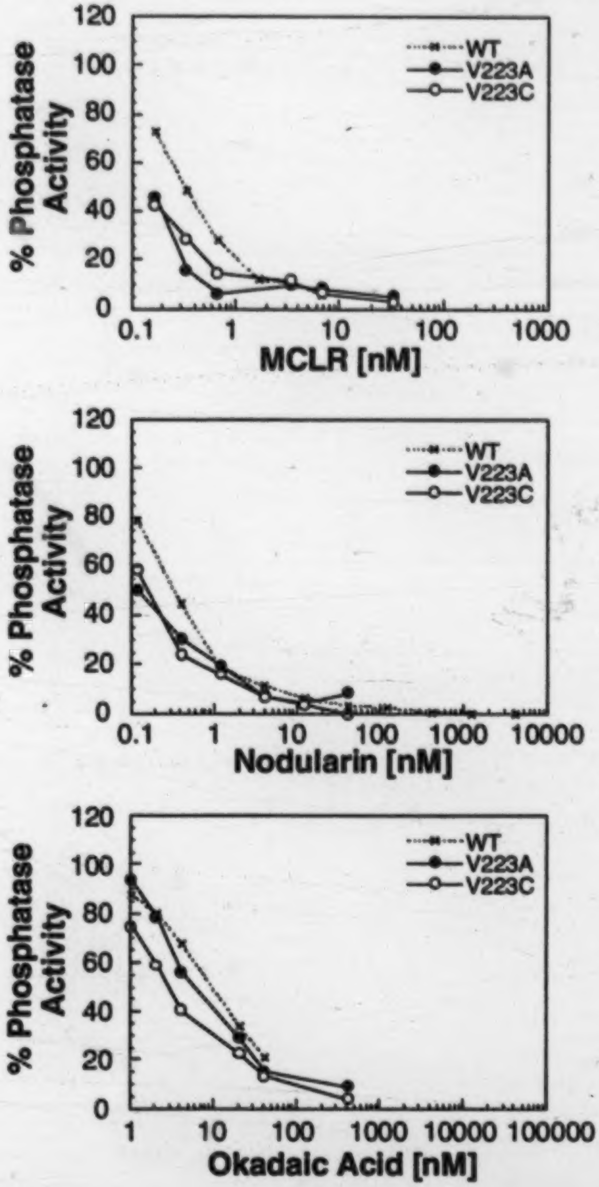
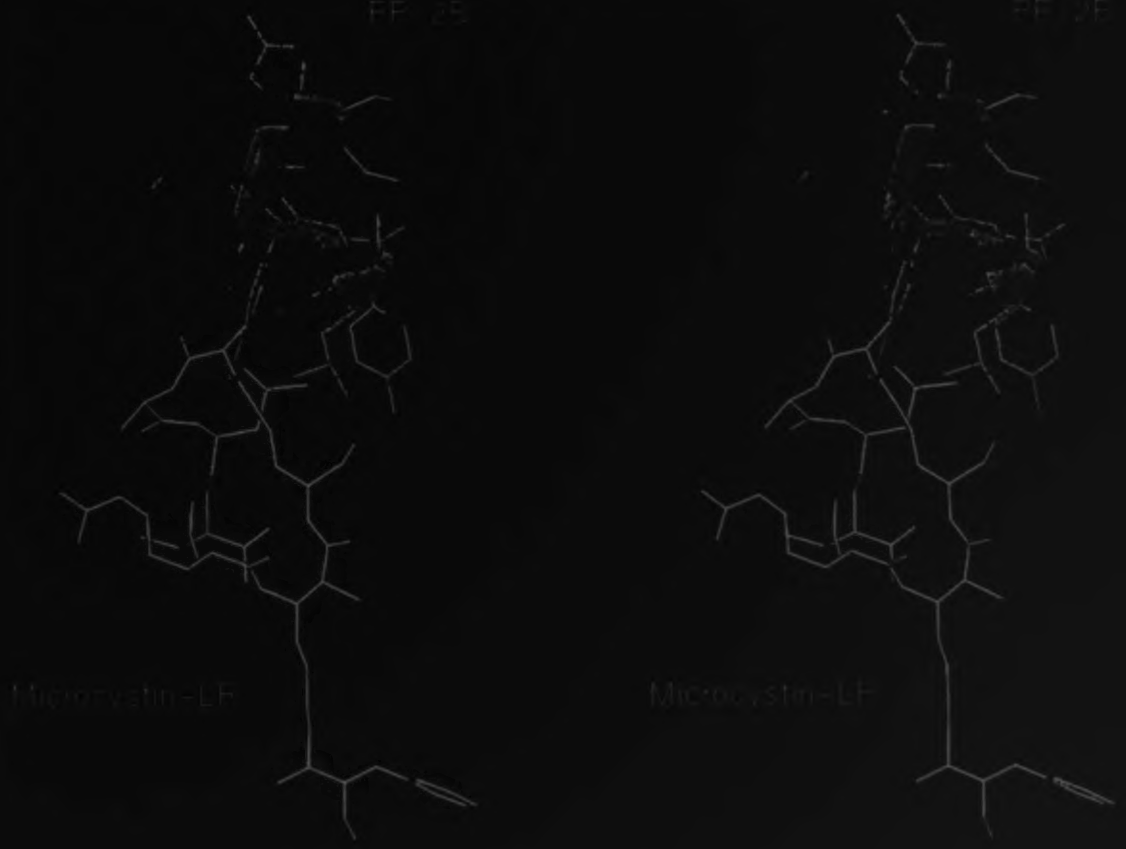


Fig. 5.16 Dose response curves of the V223 PP-1 mutants for the marine toxins. A. The dose response curve of the V223 PP-1 mutants for microcystin-LR relative to wild type enzyme. B. The dose response curve of the V223 PP-1 mutants for nodularin relative to wild type enzyme. C. The dose response curve of the V223 PP-1 mutants for okadaic acid relative to wild type enzyme. Each point represents the average of duplicate trials.

Fig. 5.17 Superimposition of the L7 loop of PP-1 (red) and PP-2B (green) relative to the structure of microcystin-LR when bound to PP-1 (blue). Van der Waals radii are shown to depict the volume taken up by each structure. This view is from within the center of the phosphatase looking out to optimize the differences between the backbone structures of PP-1 and PP-2B in the region of their L7 loops with a clearer view of the interference of Leu-312 and Tyr-315 of PP-2B with the bound structure of microcystin-LR. Selected amino acids of the PP-1 L7 loop are labeled in red, while amino acids of the PP-2B L7 loop are labeled in green. Microcystin-LR is accommodated by the L7 loop of PP-1, while the different backbone conformation of the PP-2B L7 loop blocks the access of microcystin-LR to the potential marine toxin binding site of FP-2B. In particular, Leu-312 of PP-2B interferes with the leucine sidechain of microcystin-LR while Tyr-315 completely overlaps with microcystin-LR structure. I hypothesize that the radically different backbone conformation of the L7 loop of PP-2B is responsible for the resistance of PP-2B to the marine toxins. Hydrogen atoms are not shown for clarity. This figure was generated using Insight II software.


FF 2B
Microcystin-LF
FF 2B
Microcystin-LF

I proposed that the radically different α-carbon backbone conformation between PP-1 and PP-2B is responsible in large part for the resistance of PP-2B to the marine toxins. I further hypothesized that the strikingly different backbone conformations between PP-1 and PP-2B in the region of the L7 loop were intrinsic to the amino acids of the comparable loops. Most notably, the transition from the conserved tyrosine residues, which adopt the same three-dimensional orientation in both PP-1 and PP-2B, to either Cys-273 in PP-1 or Leu-312 in PP-2B exhibits an almost 180° difference in backbone conformation between the two enzymes.

Characterization of the PP-1γ L7 Loop mutant

I felt that single amino acid substitutions of the residues in the L7 Loop mutant identified by our molecular modeling strategy would not be sufficient to emulate the backbone structural differences between PP-1 and PP-2B. I therefore mutated the entire comparable loop in PP-1 (CGEFD273-277LDVYN, referred to as the L7 Loop mutant). This mutant was the most soluble of the PP-1 mutants described in this chapter, although enough was insoluble such that the PP-1 purification procedure outlined in Chapter 4 could be employed.

Dose response curves for the Loop mutant were determined (Fig. 5.18). The curves are not dramatically different from the comparable wild type PP-1 curves. This suggests that the L7 loop of PP-2B, when present in PP-1, does not confer resistance to the marine toxins.

5.4 ■ DISCUSSION

Interactions between PP-1 and the marine toxins microcystin-LR, nodularin, and okadaic acid underlie the inhibition of the phosphatase by these compounds. The affinity of these toxins for the phosphatase is very high, such that the phosphatase is sometimes considered the cellular receptor of the toxins, forming the basis of their toxic effects. Microcystin-LR and nodularin are cyclic peptides produced by cyanobacteria in both the freshwater and marine environments that represent a threat to human health. Alternatively, the polyether fatty acid okadaic acid, which is produced by marine dinoflagellates such as *Prorocentrum lima*, is the causative agent of diarrhetic shellfish poisoning in humans.

The crystal structure of microcystin-LR bound to PP-1 was used to identify amino acid residues of PP-1 that might interact with the inhibitor. Additionally, the free structures of nodularin and okadaic acid were docked onto the crystal structure of PP-1

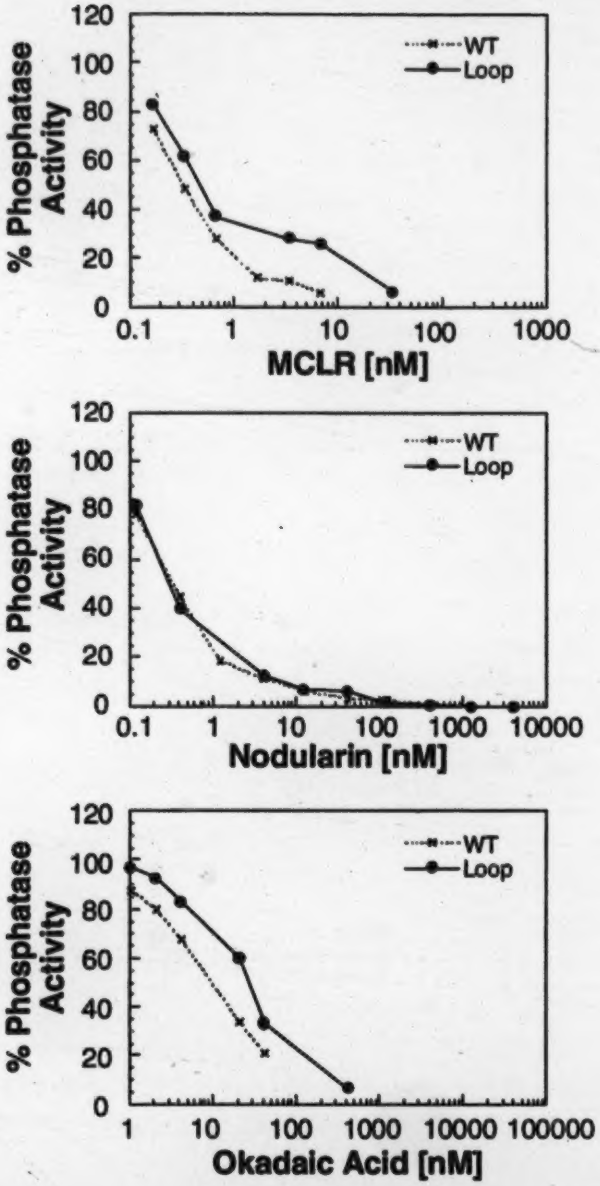


Fig. 5.18 Dose response curves of the L7 Loop PP-1 mutant for the marine toxins. A. The dose response curve of the L7 Loop PP-1 mutant for microcystin-LR relative to wild type enzyme. B. The dose response curve of the L7 Loop PP-1 mutant for nodularin relative to wild type enzyme. C. The dose response curve of the L7 Loop PP-1 mutant for okadaic acid relative to wild type enzyme. Each point represents the average of duplicate trials.

and sidechains of PP-1 identified that might influence toxin binding and inhibition [24]. Two amino acids that were candidates for toxin interaction, Trp-206 and Tyr-134, were tested for their contribution to PP-1 sensitivity to the marine toxins by substituting these residues with other amino acids and then measuring changes in marine toxin sensitivity of the resulting mutant PP-1.

Another approach to elucidating the molecular mechanism underlying PP-1 sensitivity to the marine toxins was to compare the three-dimensional structures of PP-1 and PP-2B. Despite sharing strikingly similar protein folding in their active centers, which has been identified as participating in the binding of the toxic inhibitors, PP-2B is relatively resistant to inhibition by the marine toxins. I hypothesized that this resistance is based on differences in the structure of PP-2B in its active site. We therefore employed molecular modeling to dock the bound form of microcystin-LR onto the crystal structure of PP-2B and identified seven amino acids that lie within 4Å of microcystin-LR that may encroach upon the potential toxin binding domain of PP-2B, thereby blocking entry of the toxins to the active site and conferring resistance. I then substituted these amino acids into PP-1 at their homologous position and measured changes in toxin sensitivity of the PP-1 mutants. I hypothesized that if an amino acid of PP-2B contributes to toxin resistance, then the PP-1 mutant which possesses that amino acid should exhibit increased marine toxin resistance.

Several mutant forms of PP-1 were expressed in *E. coli* as insoluble protein. I was able to exploit the insoluble PP-1 purification protocol that I developed for purification of recombinant sds21 in Chapter 4 of this thesis to purify mutant forms of PP-1 that were ordinarily expressed in insoluble form for characterization of their marine toxin sensitivity.

Three of the mutants produced possessed specific activities substantially lower than that of wild type enzyme. The W206E mutant was most severely compromised in its activity, with a specific activity almost 150 times lower than that of wild type. In addition, both of the mutations of Tyr-134, Y134F and Y134A, had lower specific activity. Both of these PP-1 amino acids are believed to be involved in recognition of the pSer or pThr of PP-1 substrates [28]. Although the goal of this work was to examine the molecular mechanism of marine toxin inhibition of PP-1 and therefore K_M values of the PP-1 mutants were not determined, the decreases in specific activity of Trp-206 and Tyr-134 PP-1 mutants support the hypothesis that they are involved in catalysis.

Several other mutants of PP-1 exhibited higher specific activity than wild type, namely the I133Y, S129H and L7 Loop mutants. This might suggest that these mutations somehow increase the activity of the enzyme. However, I suspect that this phenomenon is a function of the purification protocol employed. Recall that the wild type enzyme employed was purified from the soluble fraction of *E. coli* cell extracts while the mutants described in this chapter were purified from insoluble protein. When PP-1 is purified as insoluble protein, it undergoes a denaturation-renaturation process that involves the presence of manganese. When PP-1 is purified from the soluble fraction of *E. coli* cell lysates, I believe that a proportion of the protein expressed is inactive due to misfolding or unavailability of metal ions to bind. This inactive recombinant soluble PP-1 copurifies with active PP-1, thereby depressing the specific activity of the wild type enzyme preparation. Alternatively, when insoluble protein undergoes denaturation and renaturation, a larger proportion of the PP-1 is refolded into the active conformation. As a result, almost no inactive PP-1 is purified in this procedure.

To test this assumption, one could investigate the effect of denaturing and renaturing an aliquot of purified wild type enzyme. Comparison of the specific activities of non-renatured and renatured wild type enzyme might provide clues as to why several of the mutant forms of PP-1 purified from insoluble inclusion bodies possess specific activities greater than that of the wild type enzyme purified from soluble protein.

A challenge associated with measuring changes in marine toxin sensitivity of the various mutants was the effect of enzyme concentration on the dose response curves employing the tight binding marine inhibitors. Since several of the mutant forms of PP-1 produced possessed specific activities that were different than the wild type enzyme, different protein concentrations were employed in the assay to achieve 20% release of ^{32}P label in control assays without toxin present. As a result, levels of enzyme greater than that of wild type for the W206E, Y134F and Y134A mutants were employed. However, due to the dramatic changes in toxin sensitivity of these enzymes, enzyme concentration effects were not a major factor in the assays. Alternatively, when higher specific activity mutants were employed, the concentration of PP-1 employed was lower than that of wild type. In these cases, the dose response curves are expected to shift slightly to the left of the wild type curves, as was observed in some instances with the S129H, I133Y and V223 mutants.

The W206E mutant dose response curves were irregular in that they neither exhibited complete inhibition nor complete resistance at the inhibitor concentrations employed. These were curious results, since all of the other mutant forms of PP-1 documented thus far [22] exhibit complete inhibition or resistance over the range of toxin concentrations employed in the experiments detailed in this chapter. In addition, since other mutants of PP-1 possessing similarly compromised specific activity have also been tested and exhibit more regular dose response curves, the W206E curves can not be explained by the presence of the amount of enzyme in the assays. Therefore, I conclude that the dose response curves of the W206E mutant are due to the mutation itself.

Previous workers have suggested that hydrophobic interactions might represent the driving force behind binding of the toxins to PP-1 [24]. According to this hypothesis, the marine toxins might bind to PP-1 through a two-step process. The first step is binding of the hydrophobic regions of the toxins into the hydrophobic groove of PP-1. The second step is binding of the cyclical region of the toxins into the active site of the phosphatase. The W206A PP-1 mutant has been recently purified and characterized (Ms. H. A. Luu, pers. comm.) and shown to exhibit more regular toxin dose response curves. This suggests that the introduction of a negatively charged sidechain in the hydrophobic groove of PP-1 is responsible for the irregular dose response curves observed with the W206E mutant. Profound increases in the IC_{50} of the marine toxins with the W206A mutant, being 20000-fold larger for microcystin-LR, 10000-fold larger for nodularin, and 5000-fold for okadaic acid, supports the hypothesis that hydrophobic interactions between the marine toxins and the phosphatase in this region is a major factor contributing to the sensitivity of the phosphatase to the inhibitors.

Mutation of Tyr-134 of PP-1 resulted in enzyme with lower specific activity than the wild type. This supports the hypothesis that Tyr-134 is involved in the recognition of pSer and pThr in PP-1 substrates. The crystal structure of microcystin-LR bound to PP-1 suggests that the hydroxyl group of Tyr-134 interacts with the cyclic peptide inhibitor through a hydrogen bond. Likewise, the modeled structure of nodularin docked into PP-1 also suggests that a comparable hydrogen bond interaction occurs with the cyclic pentapeptide inhibitor. The homologous residue in PP-2B, Phe-160, was identified in our molecular modeling strategy as lying within 4Å of the docked structure of microcystin-LR, and might therefore play a role in the response of that phosphatase to the marine toxins. It was decided to examine the effect of removing the hypothesized hydrogen bond interaction with the toxins by generating the Y134F mutant of PP-1. To assess the involvement of the aromatic ring of Tyr-134, the Y134A mutant was also constructed.

The dose response curves of the Y134F mutant demonstrate that removal of the hydroxyl of Tyr-134 causes an approximately 10-fold increase in the IC_{50} for the mutant when microcystin-LR or nodularin is tested, supporting the hypothesis that a hydrogen bond is present between the inhibitors and PP-1. The dose response curve of the Y134F mutant for okadaic acid demonstrates no significant change in IC_{50}. This suggests that a hydrogen bond does not occur between PP-1 and bound okadaic acid. These data suggest that, although the marine toxins appear to bind to the same region of PP-1, they do so through subtly different interactions. For example, the cyclic peptide inhibitors utilize hydrogen bonding with Tyr-134 of PP-1 while okadaic acid does not.

Analysis of the importance of hydrophobic interactions between the inhibitors and Tyr-134 was achieved by the construction of the Y134A PP-1 mutant. When an aromatic moiety is removed from amino acid position 134 of PP-1, the IC_{50} of okadaic acid increases approximately 1500-fold. The IC_{50}'s of the cyclic peptide inhibitors also increase, with microcystin-LR increasing about 600-fold and nodularin increasing 100-fold. These dose response curves demonstrate that, once again, the different marine toxins employ similar interactions. Okadaic acid does not form a hydrogen bond with Tyr-134 of PP-1, but may rely on hydrophobic interactions with the phenyl ring for binding. Alternatively, the cyclic peptide inhibitors combine binding energy from both hydrogen bonds and hydrophobic interactions with Tyr-134 of PP-1.

Comparison of the crystal structures of PP-1 and PP-2B in this study revealed that the active site architecture of the two phosphatases was strikingly similar. As such, the fact that PP-2B is relatively resistant to the marine toxins is of interest because all of the amino acids in PP-1 shown to influence inhibitor efficiency in this work and previous studies [22] are conserved in PP-2B. Therefore, PP-2B appears to possess a potential toxin binding site that contains all of the amino acids required for high affinity binding of the marine toxins. I proposed that structural differences between PP-1 and PP-2B in the region of the toxin binding site of PP-1 account for the resistance of PP-2B to the marine toxins.

We employed molecular modeling to dock the bound structure of microcystin-LR onto the crystal structure of PP-2B in the region of PP-2B that is homologous to the toxin binding region of PP-1. Seven amino acids were identified within 4Å of the docked microcystin-LR that were different from their PP-1 homologs and might block the access of the marine toxins to the potential toxin binding region of PP-2B. To analyze the contribution of these amino acids to PP-2B marine toxin resistance, I substituted them into PP-1 and measured the dose response curves of the resulting mutants. My hypothesis was that if a particular amino acid in PP-2B was blocking access to the potential toxin binding region of PP-2B, then substituting the homologous amino acid in PP-1 with the PP-2B residue should result in increased toxin resistance of the mutant.

The contribution of His-155 to PP-2B marine toxin resistance was analyzed by mutating the homologous Ser-129 of PP-1. The S129H PP-1 mutant had a higher specific activity than the wild type enzyme. As a result, if the dose response of the S129H mutant is unchanged, then we would expect to see a slight shift in the dose response curve to the left of the wild type curve to account for the lower concentration of enzyme in the assays.

This is the case with the nodularin dose response curve. In the case of microcystin-LR and okadaic acid, however, the dose response curves are shifted slightly to the right of the wild type curves, suggesting modest increases in resistance against these two marine toxins. This suggests that His-155 may interact in a minor way with microcystin-LR and okadaic acid, but certainly not enough to account for the relative resistance of PP-2B to the toxins. Alternatively, these results suggest that Ser-129 of PP-1 may contribute slightly to the high affinity binding of microcystin-LR and okadaic acid, but not nodularin.

Tyr-159 of PP-2B might block access to the potential toxin binding region of PP-2B. The comparable amino acid in PP-1 is Ile-133, which is smaller and does not interfere with toxin binding in PP-1. I therefore mutated Ile-133 of PP-1 to tyrosine and tested the resulting I133Y PP-1 mutant for changes in marine toxin sensitivity. This mutant possessed the highest specific activity of all the mutants purified, being five times higher than wild type. As a result, we would expect that the dose response curves for this mutant would be shifted to the left of the wild type curves if the mutation causes no change in toxin sensitivity due to enzyme concentration effects in the assays. This occurs in the cases of microcystin-LR and nodularin. However, when the response to okadaic acid is measured, an increase in IC_{50} occurs. This difference could be as much as a 10-fold increase in IC_{50}. Therefore, it appears that Tyr-159 of PP-2B may contribute to resistance of PP-2B to okadaic acid. Conversely, the presence of Ile-133 may accommodate okadaic acid in the toxin binding region of PP-1.

The contribution of Cys-256 of PP-2B to the resistance of the phosphatase to marine toxins was analyzed by mutations of the homologous residue of PP-1 (Val-223). These results failed to provide any evidence that these amino acids significantly contribute to the resistance of PP-2B or sensitivity of PP-1 to the marine toxins.

In summary, all of the evidence gathered to this point strongly suggests that, although the marine toxins appear to bind to PP-1 at the same site, they do so through slightly different mechanisms. While all seem to rely on hydrophobic interactions meditated by Trp-206, only the cyclic peptide inhibitors interact with the hydroxyl group of Tyr-134 through a hydrogen bond. All of the marine toxins interact with PP-1 through hydrophobic contacts mediated by the aromatic ring of Tyr-134, but the binding of okadaic acid is more dependent on this interaction than the cyclic peptide inhibitors. Similarly, the cyclic peptide inhibitors are unaffected by substituting a larger tyrosine residue for Ile-133, while the larger tyrosine residue appears to occlude the binding of okadaic acid in the toxin binding region to a small degree. The binding of both microcystin-LR and okadaic acid was disrupted slightly by substitution of Ser-129 with histidine, while the smaller inhibitor nodularin was unaffected by this change.

My data also suggest that PP-2B possesses amino acids in its active site that are amenable to toxin binding. Only slight increases in IC_{50} occur in PP-1 mutants possessing homologous PP-2B amino acids for microcystin-LR and okadaic acid. Of particular interest is the observation that none of the PP-1 mutations based on our docking of microcystin-LR onto the potential toxin binding region of PP-2B increased the resistance of the mutants to nodularin. To my knowledge, the IC_{50} of nodularin for PP-2B has not been determined. It is possible that the smaller nodularin might actually inhibit PP-2B to a reasonable degree when compared to okadaic acid and microcystin-LR.

Since it appears that none of the amino acids involved in the active site of PP-2B are responsible for the resistance of this phosphatase to the marine toxins, other structural features of PP-2B must be involved. The remaining three amino acids identified in PP-2B as lying within 4Å of the docked microcystin-LR structure are all found within the L7 loop of PP-2B. The possibility that the L7 loop of PP-2B contributes to resistance of the phosphatase to marine toxins is a logical hypothesis, since the L7 loop of PP-1 has already been identified as playing a role in decreased sensitivity of that phosphatase to okadaic acid relative to PP-2A [11]. We therefore took a closer look at the three-dimensional structures of PP-1 and PP-2B in the region surrounding the L7 loops of the two phosphatases and observed that the two loops possess radically different α-carbon backbone conformations. After a conserved tyrosine (Tyr-272 of PP-1 and Tyr-311 of PP-2B, which is involved in metal binding in the active site), which have similar orientations, the backbones of the two L7 loops are directed in almost precisely opposite directions. Following five amino acids, the two loops rejoin at a conserved asparagine residue and follow essentially the same three-dimensional orientation until the end of the PP-1 crystal structure, at which point the PP-2B structure continues to the C-terminal α-helix present only in PP-2B. Two of the PP-2B L7 loop amino acids, Leu-312 and Tyr-315 appear to jut into the potential toxin binding region of PP-2B. The comparable sidechains of PP-1 are directed away from the toxin binding pocket of that phosphatase such that the marine toxins are accommodated.

I proposed that the different L7 loop conformation of PP-2B may be responsible for the resistance of the phosphatase to the marine toxins. I also hypothesized that the conformation of the PP-2B L7 loop was intrinsic to the amino acids of the loop itself. Based on this idea, I reasoned that generating single substitution mutants of PP-1 to emulate PP-2B in the region of the L7 loop would not be sufficient to alter the backbone structure of the loop. I therefore substituted all five amino acids of the PP-1 L7 loop with the amino acids of the PP-2B L7 loop. The resulting mutant, referred to here as the L7 Loop mutant, was then tested for any changes in toxin sensitivity.

I found no significant increase in resistance to the marine toxins with the L7 Loop mutant. This finding raises the question: What is the conformation of the L7 loop in the mutant PP-1? Based on our structural modeling, it would be reasonable to hypothesize that the backbone of the L7 loop in the mutant PP-1 conforms to that of PP-1 rather than PP-2B. A corollary of this hypothesis is that in the PP-1 conformation, the amino acids of the L7 loop of PP-2B do not confer resistance to the marine toxins.

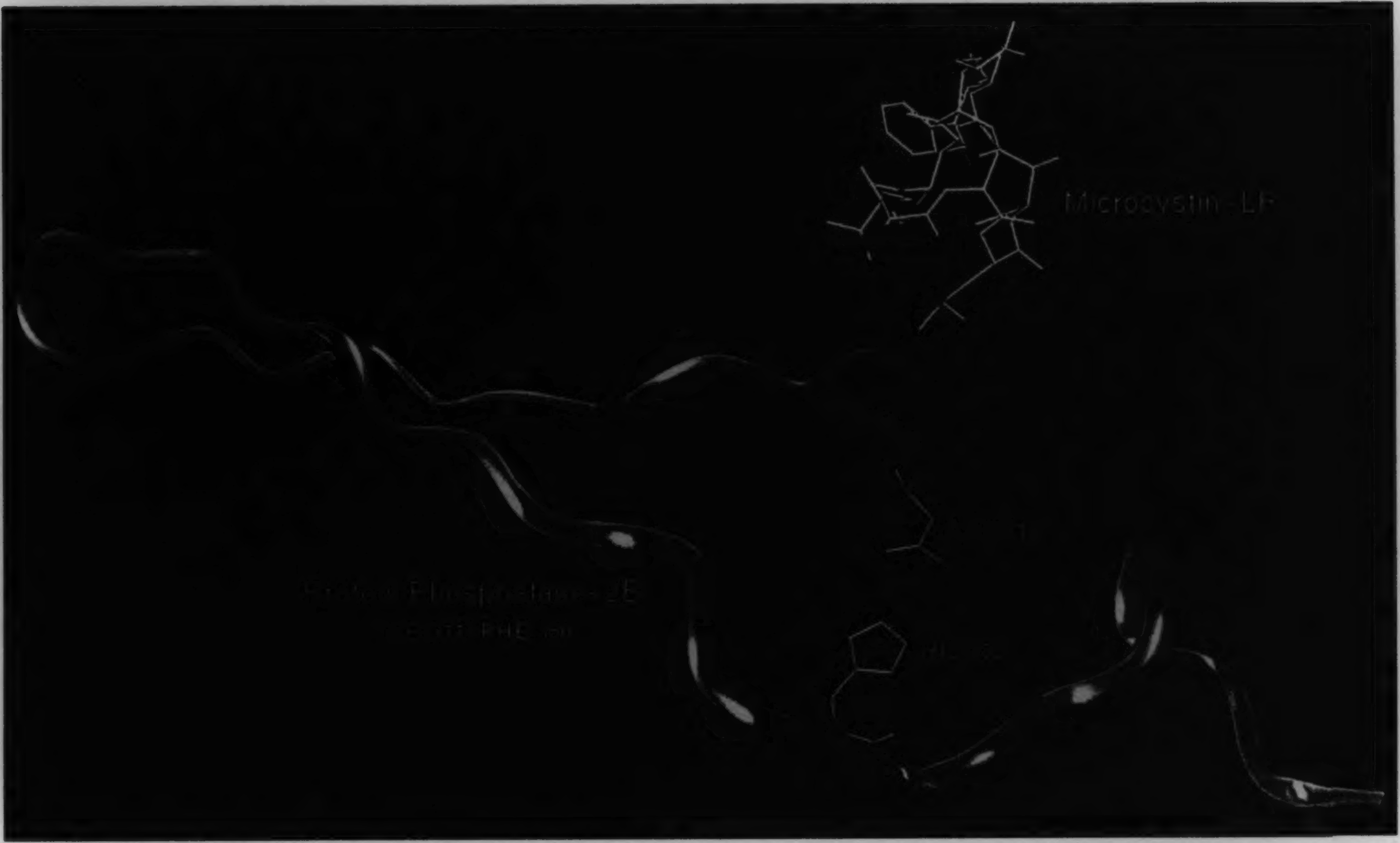
Since I believe that the L7 loop in the mutant PP-1 possesses the PP-1 backbone structure, I hypothesized that there must exist another structural feature in PP-2B that is lacking in PP-1 and is responsible for the radically different L7 configuration of PP-2B. The most logical candidate for such a structure would be within the C-terminal tail of PP-2B which is completely absent from PP-1.

Closer examination of the crystal structure of PP-2B revealed that His-339 of the C-terminal tail is closely associated with Asp-313 of the L7 loop (Fig. 5.19). This association may be responsible for the L7 backbone conformation. Since the C-terminal tail of PP-2B is lacking in PP-1, the PP-1 L7 Loop mutant does not possess a homologous His-339 and is therefore not constrained to adopt the conformation of the L7 loop in PP-2B. Without the confining association with His-339, the PP-1 L7 Loop mutant is free to adopt the conformation of the native PP-1 loop and in this conformation does not confer resistance to the marine toxins to the mutant PP-1.

I therefore propose that association with His-339 constrains the L7 loop into a conformation that blocks marine toxins from entering the catalytic site of PP-2B. This hypothesis is supported by the observation that the L7 loop of PP-1 undergoes a slight structural shift to accommodate the leucine sidechain of microcystin-LR as it packs against Tyr-272 of PP-1 [20]. By fixing the conformation of the L7 loop of PP-2B, the flexibility required to accommodate the marine toxins is lost. Unfortunately, since PP-1 does not possess a comparable residue to His-339 of PP-2B, we are unable to test this hypothesis using our PP-1 mutagenesis system. Mutation of PP-2B itself is required.

Wei and Lee [29] have produced mutants of PP-2B in the L7 loop where the L7 loops of PP-1 (GEFD) and PP-2A (YRCG) were inserted into PP-2B. Increased activity of these calcineurin mutants against *p*NPP and the R_{II} peptide was observed. In their discussion, Wei and Lee note that the activity of PP-2B seems to be constrained by two elements: the autoinhibitory domain and something to do with the structure of the L7 loop. Wei and Lee, however, provide no structural hypothesis for the observed changes.

Fig. 5.19 His-339 of PP-2B interacts with Asp-313 of the L7 loop. Depicted is a superimposition of the homologous C-terminal regions of PP-1 (red) and PP-2B (green), beginning at the conserved Tyr residues of the L7 loops, with the structure of microcystin-LR when bound to PP-1 (blue). While amino acids after Ala-300 of PP-1 are unstructured, the C-terminal tail of PP-2B continues into the C-terminal α-helix which contains the binding domains of calmodulin and the B subunit of PP-2B. Within the linker region of PP-2B between the catalytic domain and the C-terminal helix is His-339, which appears to interact with Asp-313 of the L7 loop of PP-2B, thereby constraining the conformation of the L7 loop such that it is "pulled down" in this orientation relative to the L7 loop of PP-1. A homologous interaction is not possible in PP-1, since its C-terminal tail is unstructured. I hypothesize that the interaction of His-339 and Asp-313 of PP-2B is responsible for the radically different L7 loop backbone conformation that confers resistance to the marine toxins. Hydrogen atoms are not shown for clarity. This figure was generated using Insight II software.



Microcystin - LF
Protein Phosphatase 2B

My hypothesis that the histidine-aspartic acid interaction constrains the L7 loop resulting in toxin blockage might also explain Wei and Lee's results. By mutating the L7 loop such that the interacting Asp-313 is mutated to glycine (PP-1) or tyrosine (PP-2A), Wei and Lee have removed the histidine-aspartic acid interaction. The result is an unrestricted active site that exhibits increased activity. It is interesting to note that the PP-1 mutant with glycine in place of Asp-313 possesses greater activity than the comparable PP-2A mutant with a tyrosine replacement. Since tyrosine has greater polar character than glycine, perhaps some minor interaction with His-339 accounts for these differences.

According to my predictions, the PP-2B L7 Loop mutants produced by Wei and Lee should possess unconstrained L7 loops. It will therefore be a future interest to test the resistance of these mutants to the marine toxins. It will also be important to produce mutants of PP-2B in which the His-339 or Asp-313 have been specifically mutated to test the hypothesis put forth here. Since it is suggested that the L7 loop of PP-2B does not confer resistance to the marine toxins when in an unconstrained conformation, such as that in the L7 Loop mutant of PP-1, simply mutating the His-339 may be enough to produce a form of PP-2B that exhibits greater sensitivity to the marine toxins.

The aim of the research presented in this chapter was to determine the molecular mechanism of PP-1 inhibition by the marine toxins. The evidence gathered here supports the idea that the marine toxins bind PP-1 at the same site and that hydrophobic interactions play a major role in high affinity binding of marine toxins to that site. My results also extend this hypothesis by suggesting that, although the marine toxins may bind at the same site, they do so through subtly different interactions, reflecting the disparate chemical structures of the inhibitors. It remains to characterize the precise set of interactions for each marine toxin to produce an accurate map of the specific interactions of each marine toxin with the PP-1 toxin binding region.

Moreover, my data also suggest that PP-2B possesses a potential toxin binding region homologous to PP-1, containing residues that are amenable to inhibition by the marine toxins. However, dramatic structural differences in the L7 loop of PP-2B conspire to block access to this potential toxin binding region of PP-1 by the marine toxins. This hypothesis satisfactorily explains a problem that has puzzled protein phosphatase researchers for some time: Why are PP-1 and PP-2A so sensitive to inhibition by the marine toxins while PP-2B is not? In the future, it will be important to test this hypothesis through mutagenesis of the catalytic subunit of PP-2B. If the major structural feature of PP-2B that is responsible for marine toxin resistance is identified, we may be able to alter its structure such that a form of PP-2B that is sensitive to inhibition by the marine toxins is produced. Then we can move forward to investigate the contribution of the finer structural differences between PP-1 and PP-2B that seem to contribute in minor ways to the resistance of PP-2B to the marine toxins. The amino acids identified in our docking of microcystin-LR onto the structure of PP-2B would be good candidates for such "fine-tuning" of the potential PP-2B toxin binding site.

In addition, a form of PP-2B that is sensitive to the marine toxins would be a valuable research tool in the elucidation of physiological processes governed by protein serine/threonine phosphatases. Altering PP-2B in a cell line such that it is sensitive to the marine toxins would permit inhibition of three of the four major protein serine/threonine phosphatases in eukaryotic cells. This could be used to identify new substrates and determine novel physiological roles for PP-2B and indirectly contribute to examining PP-2C activity *in vivo*.

5.5 ■ BIBLIOGRAPHY

1. Hunter, T., and Plowman, G. D. (1997) The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.* **22**: 18-22.

2. Holmes, C. F. B., Luu, H. A., Carrier, F., and Schmitz, F. J. (1990) Inhibition of protein phosphatases-1 and -2A with acanthifolicin. *FEBS Lett.* **270**: 216-218.

3. Nishiwaki, S., Fujiki, H., Suganuma, M., Furuya-Suguri, H., Matsushima, R., Iida, Y., Ojika, M., Yamada, K., Uemura, D., Yasumoto, T., Schmitz, F. J., and Sugimura, T. (1990) Structure-activity relationship within a series of okadaic acid derivatives. *Carcinogenesis* **11**: 1837-1841.

4. Stotts, R. R., Namikoshi, M., Haschek, W. M., Rinehart, K. L., Carmichael, W. W., Dahlem, A. M., and Beasley, V. R. (1993) Structural modifications imparting reduced toxicity in microcystins from *Microcystis* spp. *Toxicon* **31**: 783-789.

5. Rinehart, K. L., Namikoshi, M., and Choi, B. W. (1994) Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). *J. Appl. Phycol.* **6**: 159-176.

6. Nishiwaki-Matsushima, R., Nishiwaka, S., Ohta, T., Yoshizawa, S., Suganuma, M., Harada, K., Watanabe, M. F., and Fujiki, H. (1992) Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatases. *Jpn. J. Cancer Res.* **82**: 993-996.

7. Craig, M., McCready, T. L., Luu, H. A., Smillie, M. A., Dubord, P., and Holmes, C. F. B. (1993) Identification and characterization of hydrophobic microcystins in Canadian freshwater cyanobacteria. *Toxicon* **31**: 1541-1549.

8. Stone, S. R., Mayer, R., Wernet, W., Maurer, F., Hofsteenge, J., and Hemmings, B. A. (1988) The nucleotide sequence of the cDNA encoding the human lung protein phosphatase 2A α catalytic subunit. *Nucleic Acids Res.* **16**: 11365.

9. Griffith, J. P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. T., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. A. (1995) X-ray structure of calcinuerin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell* **82**: 507-522.

10. Shima, H., Tohda, H., Aonuma, S., Nakayasu, M., DePaoli-Roach, A. A., Sugimura, T., and Nagao, M. (1994) Characterization of the PP2Aα gene mutation in okadaic acid-resistant variants of CHO-K1 cells. *Proc. Natl. Acad. Sci. USA* **91**: 9267-9271.

11. Zhang, Z., Zhao, S., Long, F., Zhang, L., Bai, G., Shima, H., Nagao, M., and Lee, E. Y. C. (1994) A mutant of protein phosphatase-1 that exhibits altered toxin sensitivity. *J. Biol. Chem.* **269**: 16997-17000.

12. Yoshizawa, S., Matsushima, R., Watanabe, M. F., Harada, K. I., Ichihara, K., Carmichael, W. W., and Fujiki, H. (1990) Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. *J. Cancer Res. Clin. Oncol.* **116**: 609-614.

13. Matsushima, R., Yoshizawa, S., Watanabe, M. F., Harada, K., Mitsuru, F., Carmichael, W. W., and Fujiki, H. (1990) *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin fibroblasts. *Biochem. Biophys. Res. Comm.* **171**: 867-874.

14. Zhang, L., Zhang, Z., Long, F., and Lee, E. Y. C. (1996) Tyrosine-272 is involved in the inhibition of protein phosphatase-1 by multiple toxins. *Biochemistry* **35**: 1606-1611.

15. MacKintosh, R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T. W., Cohen, P., and MacKintosh, C. (1995) The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Lett.* **371**: 236-240.

16. Craig, M., Luu, H. A., McCready, T. L., Williams, D. E., Andersen, R. J., and Holmes, C. F. B. (1996) Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. *Biochem. Cell. Biol.* **74**: 569-578.

17. Goldberg, J., Huang, H., Kwon, Y., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **376**: 745-753.

18. Kissinger, C. R., Parge, H. E., Knighton, D. R., Lewis, C. T., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, R. E., Moomaw, E. W., Gastinel, L. N., Habuka, N., Chen, X., Maldonado, F., Barker, J. E., Bacquet, R., and Villafranca, J. E. (1995) Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. *Nature* **378**: 641-644.

19. Bagu, J. B., Sonnichsen, F. D., Williams, D. E., Andersen, R. J., Sykes, B. D., and Holmes, C. F. B. (1995) Comparison of the solution structures of microcystin-LR and motuporin. *Nature Struct. Biol.* **2**: 114-116.

20. Barford, D. (1996) Molecular mechanisms of the protein serine/threonine phosphatases. *Trends Biochem. Sci.* **21**: 407-412.

21. Zhang, L., and Lee, E. Y. C. (1997) Mutational analysis of substrate recognition by protein phosphatase 1. *Biochemistry* **36**: 8209-8214.

22. Huang, H.-B., Horiuchi, A., Goldberg, J., Greengard, P., and Nairn, A. C. (1997) Site-directed mutagenesis of amino acid residues of protein phosphatase 1 involved in catalysis and inhibitor binding. *Proc. Natl. Acad. Sci. USA* **94**: 3530-3535.

23. Quinn, R. J., Taylor, C., Suganuma, M., and Fujiki, H. (1993) The conserved acid binding domain model of inhibitors of protein phosphatases 1 and 2A: molecular modelling aspects. *Bioorg. Med. Chem. Lett.* **3**: 1029-1034.

24. Bagu, J. R., Sykes, B. D., Craig, M. M., and Holmes, C. F. B. (1997) A molecular basis for different interactions of marine toxins with protein phosphatase-1. *J. Biol. Chem.* **272**: 5087-5097.

25. Ohta, T., Sueoka, E., Iida, N., Komori, A., Suganuma, M., Nishiwaki, R., Tatematsu, M., Kim, S.-J., Carmichael, W. W., and Fujiki, H. (1994) Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Res.* **54**: 6402-6406.

26. Barford, D., and Keller, J. C. (1994) Co-crystallization of the catalytic subunit of the serine/threonine protein phosphatase 1 from human in complex with microcystin LR. *J. Mol. Biol.* **235**: 763-766.

27. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-252.

28. Egloff, M.-P., Cohen, P. T. W., Reinemer, P., and Barford, D. (1995) Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.* **254**: 942-959.

29. Wei, Q., and Lee, E. Y. C. (1997) Mutagenesis of the L7 loop connecting β strands 12 and 13 of calcineurin: evidence for a structural role in activity changes. *Biochemistry* **36**: 7418-7424.

Chapter 6

General Discussion and Conclusions

6.1 ■ INTRODUCTION

Protein phosphatase-1 (PP-1) is one of only four major serine/threonine phosphatases acting in eukaryotic cells, accounting for the majority of phosphatase activity in whole cell lysates. PP-1 shares significant sequence identity with two other protein serine/threonine phosphatases, PP-2A and PP-2B, that together form the phosphoprotein phosphatase gene family, known as the PPP family. These three phosphatases can be distinguished by several biochemical means (see Table 1.2), among them differential sensitivity to a growing family of diverse marine toxins. These include the polyether fatty acid okadaic acid, produced by marine dinoflagellates such as *Prorocentrum lima*, and cyclic peptides of the microcystin and nodularin classes, produced in both the marine and freshwater environments by cyanobacteria.

These toxins bind and inhibit PP-1 with such high affinity (in the nM range) that PP-1 is often considered an intracellular receptor of the toxins, mediating their toxic effects. Since PP-1 is a key regulator of a wide variety of physiological processes governing the dephosphorylation of many proteins on serine and threonine residues, inhibition of this enzyme by the marine toxins can clearly have detrimental consequences. Intoxication with okadaic acid causes diarrhetic shellfish poisoning, while high doses of microcystins or nodularins can cause lethal liver damage.

Understanding the regulation of PP-1 is key to a fuller appreciation of the complexities of signal transduction involving reversible protein phosphorylation. In addition, elucidating the mechanism by which PP-1 is inhibited by the marine toxins will facilitate a clearer understanding of the toxic effects of these natural-product inhibitors.

PP-1 is regulated by several mechanisms which may operate in a combined manner. Among these is the association of the PP-1 catalytic subunit with other proteins. This association serves to confine PP-1 activity to a specific subcellular location or alter its catalytic efficiency or substrate affinity. Phosphorylation of these associating proteins often influences their interactions with PP-1.

Recently, it has been demonstrated that phosphorylation of the catalytic subunit of PP-1 itself also represents another mode of regulation of PP-1 activity. Specifically, the phosphorylation of Thr-320 of several PP-1 isoforms by cdc2 kinase reduces the activity of PP-1. Since the activity of cdc2 kinase fluctuates during the cell cycle, the regulation of PP-1 activity by phosphorylation of Thr-320 is likewise cell cycle-dependent. Other evidence suggested that PP-1 among lower eukaryotes may be regulated by cAMP-dependent protein kinase (PKA). PKA is of particular interest because its effects are often antagonized by PP-1 activity.

PP-1 is also regulated by the marine toxins through direct interaction and inhibition of the catalytic subunit. The objective of this thesis was therefore to understand the mechanisms by which PP-1 is regulated by reversible protein phosphorylation and interaction with the marine toxins.

6.2 ■ ANALYSIS OF PROTEIN PHOSPHORYLATION BY CAPILLARY ELECTROPHORESIS

Since PP-1 is a key player in the process of reversible protein phosphorylation in eukaryotic cells, the ability to measure this process is central to experimental strategies aimed at delineating signal transduction mechanisms involving phosphorylation. In Chapter 2 of this thesis, I outline the development of a capillary electrophoresis-based assay for protein phosphorylation employing peptide fragments of *in vivo* protein serine/threonine kinase substrates. I selected protein kinases and kinase substrates that had bearing on PP-1-regulated systems. First among these was PKA, the activity of which is antagonized directly by PP-1 activity in such diverse physiological processes as glycogen metabolism and the regulation of gene expression. Employing a peptide fragment of pyruvate kinase known as Kemptide, which contains a consensus sequence for PKA phosphorylation, I demonstrated that capillary electrophoresis analysis of protein kinase reactions including Kemptide could clearly resolve phosphorylated peptide from unphosphorylated peptide. The percentage of total peptide in these reactions that was phosphorylated could be calculated since both phosphorylated and unphosphorylated peptides could be detected. Thus, capillary electrophoresis permitted direct assessment of the progress of peptide phosphorylation in the reaction mixture.

Moreover, protein kinase activity could be detected in complex mixtures such as tissue lysates by the detection of phosphorylated and unphosphorylated peptides in such reactions in the presence of appropriate kinase agonists. As a result, protein kinase activity associated with the phosphorylation of specific sites on peptides can be isolated and quantitated apart from background compounds in complex mixtures. This is a significant advance compared to other rapid protein kinase assay systems which often involve measuring incorporated isotopically-labeled phosphate onto peptide substrates. With these assay systems, only the product of the kinase reaction (isotopically-labeled peptide) can be quantitated. Therefore, direct and accurate measurement of the percentage of total peptide that is phosphorylated in a protein kinase reaction is not possible. Alternatively, with the capillary electrophoresis-based assay, both substrates and products of kinase reactions can be quantitated simultaneously.

Since most phosphoproteins are phosphorylated on multiple sites, it was important to determine if the capillary electrophoresis-based assay I developed was capable of analyzing multisite phosphorylation. In this case, the phosphorylation of a fragment of the G subunit of the PP-1G complex which regulates PP-1 activity during glycogen metabolism was investigated. This peptide, known as the PP-1G peptide, is phosphorylated on two different sites by PKA and casein kinase-II (CK-II). Analysis of simultaneous phosphorylation reactions including PKA and CK-II employing the capillary electrophoresis assay at pH 2.0 demonstrated that this procedure is able to resolve peptides phosphorylated to different degrees (i.e., unphosphorylated, phosphorylated on one site, or phosphorylated on two sites), owing to the increase in negative charge on the peptides associated with covalently bound phosphate groups.

When the pH of the analysis conditions was increased to 5.0, the amount of time required to complete the analysis was reduced due to increased electroosmotic flow within the capillary which, at pH 2.0, is negligible thereby maximizing resolution of peptides based on their net charge. However, at pH 5.0, resolution of peptides phosphorylated once on different sites was possible, in addition to resolution of peptides that were unphosphorylated or phosphorylated on both sites. Thus, capillary electrophoresis permits not only the quantitation of phosphorylation stoichiometry, but also the differentiation of peptides that have been phosphorylated to the same degree at distinct sites. This is a significant advance in protein kinase assays, since the most common protein kinase assay systems, involving isotopically-labeled phosphate, are unable to distinguish between substrates phosphorylated to the same degree at different sites. Since multisite phosphorylation occurs on most phosphoproteins *in vivo*, capillary electrophoresis analysis represents a step forward in the elucidation of the role of phosphorylation at specific sites in situations where multisite phosphorylation of a substrate is occurring.

Investigation of the Hierarchical Phosphorylation Hypothesis

An example of multisite phosphorylation that has *in vivo* significance is the phosphorylation of glycogen synthase, a key regulator of glycogen metabolism and *in vivo* substrate of PP-1, by CK-II and glycogen synthase kinase-3 (GSK-3). Since the phosphorylation consensus sequence for GSK-3 contains a pSer, prior phosphorylation by CK-II is required for GSK-3 to efficiently phosphorylate glycogen synthase. The first GSK-3 phosphorylation event creates, by definition, another GSK-3 consensus sequence, such that glycogen synthase is phosphorylated on four serine residues processively. This process is referred to by previous workers as hierarchical phosphorylation, since each phosphorylation event is dependent on the previous phosphorylation. I employed my capillary electrophoresis assay to test the hypothesis that hierarchical phosphorylation was occurring on a fragment of glycogen synthase by GSK-3. I determined that in the presence of CK-II, single peaks of different degrees of phosphorylation of the peptide were present. Alternatively, in the absence of CK-II, less phosphorylation of the peptide was observed and multiple forms representing different degrees of peptide phosphorylation were detected. This strongly suggests that prior phosphorylation by CK-II is required for efficient phosphorylation of glycogen synthase by GSK-3 and that GSK-3 phosphorylates glycogen synthase in a processive manner, supporting the hierarchical phosphorylation hypothesis.

There is great potential for the application of capillary electrophoresis to the analysis of protein phosphorylation. The protocol I developed allows the simultaneous detection of both substrate and product of phosphorylation reactions and permits the resolution of different kinase activities acting on the same substrate. When coupled with laser-induced fluorescence (LIF) detection systems, which was demonstrated in this thesis to increase the limit of detection approximately 500-fold over standard UV optics, capillary electrophoresis has the potential to be more accurate and detect smaller amounts of activity than the most commonly employed procedures today. In the future, the development of phosphorylation-specific derivatizing agents, such as those developed to specifically detect phosphoserine-containing peptides [1], for LIF detection may allow the detection and assay of very small amounts of protein kinase activity in complex mixtures. However, due to the high cost of LIF detection systems and the specialized equipment associated with capillary electrophoresis, this technique has not been widely exploited and may remain a specialized approach.

This procedure was applied in other systems to test the phosphorylation of other substrates or to detect protein serine/threonine kinases in novel systems. As such, the capillary electrophoresis-based assay augmented more traditional techniques of protein kinase detection throughout the work described in this thesis.

6.3 ■ PROTEIN KINASES AND PHOSPHATASES IN MARINE DINOFLAGELLATES

We hypothesized that investigating signal transduction involving reversible protein phosphorylation in an organism that is a primary producer of one of the marine toxins might provide insight into the mechanism of PP-1 regulation by interaction with the marine toxins. To test this hypothesis, we chose to study the marine dinoflagellate *Prorocentrum lima*. This unicellular organism is a primary producer of okadaic acid.

The choice of a marine dinoflagellate for biochemical study brought with it significant technical and theoretical challenges. Extensive biochemical characterization of marine dinoflagellates had not been performed previously. In addition, marine dinoflagellates grow slowly relative to other commonly studied organisms, with a doubling time of approximately five days. Thus, a constant challenge was obtaining significant amounts of biomass to study. Another challenge was determining the best method for producing a cell lysate with these armor-bearing cells. Initial work performed by a postdoctoral fellow in the laboratory involved sonication of cells to obtain cell lysates and thus this technique was applied in my initial work.

The Mesokaryotic Hypothesis

The phylogenetic position of dinoflagellates is controversial. Initial characterization of dinoflagellates suggested that they possessed nuclear characteristics more in line with prokaryotic features than eukaryotic. Therefore, previous investigators thought that dinoflagellates might represent an intermediate form of life between prokaryotic and eukaryotic organisms, called mesokaryotic. The mesokaryotic hypothesis was widely accepted until more recently, when more detailed analysis of the dinoflagellate nucleus and RNA sequencing suggested that dinoflagellates are true eukaryotes that have secondarily lost the genes encoding histones, thereby accounting for the unusual chromosomal characteristics of these organisms.

Investigation of the Marine Toxin Resistance Hypothesis

The mesokaryotic hypothesis was relevant to my work. To account for the apparent resistance of marine dinoflagellates to the okadaic acid they produce, some researchers hypothesized that they possess signaling molecules of a different origin than those in eukaryotes. The apparent lack of protein serine/threonine phosphatases (and phosphoserine and phosphothreonine signaling for that matter) in prokaryotes was extended to dinoflagellates. The fact that dinoflagellates that produce okadaic acid exhibit no ill effects from this very potent toxin was seen as evidence that they must be of a different evolutionary origin than eukaryotes. Therefore, any information I might gather regarding the nature of signal transduction in marine dinoflagellates would not only represent the first direct biochemical data collected for dinoflagellates in this regard, but would also facilitate assessment of the mesokaryotic hypothesis.

Therefore, as outlined in Chapter 3 of this thesis, I characterized an autophosphorylating protein tyrosine kinase from *Prorocentrum lima*, which I referred to as PLIK 1A (*P. lima* kinase 1A). The presence of a tyrosine kinase suggested that phosphotyrosine was being employed by dinoflagellates as a facet of its arsenal of regulatory mechanisms. If this hypothesis was correct, then regulated dephosphorylation of phosphotyrosine must also occur in dinoflagellates. Therefore, I searched for protein tyrosine phosphatases in *P. lima* and characterized two such activities, referred to as PLPTP-I and PLPTP-II (*P. lima* protein tyrosine phosphatases-I and II). Characterization of these activities suggested that they are two distinct enzymes based on their differential sensitivity to classical protein tyrosine phosphatase inhibitors. In addition, it was demonstrated that PLPTP-I and PLPTP-II are capable of dephosphorylating isotopically-autophosphorylated PLIK 1A.

This work was clearly an advance in our understanding of marine dinoflagellate biochemistry. My work provided the first direct evidence that marine dinoflagellates possess phosphotyrosine and the signaling molecules in the form of protein tyrosine kinases and phosphatases to employ phosphotyrosine for the regulation of biochemical processes. Moreover, my characterization of PLIK 1A as a substrate for PLPTP-I and PLPTP-II represented the first identification of a physiological phosphatase substrate in a dinoflagellate.

Following the identification and characterization of PP-1 and PP-2A activity in marine dinoflagellates by a postdoctoral fellow in the laboratory [2,3], characterization of protein serine/threonine kinases in *Prorocentrum lima* continued. Although this work initially lead to the identification of PLIK 1A, I was unable to uncover any protein serine/threonine kinase activity in cell lysates. Methods of producing cell lysates other than sonication of cells were explored and cell lysates produced with a french press were tested for the presence of casein and histone kinase activity. In these lysates, casein and histone kinase activity was present. This encouraging result lead to the identification and characterization of PKA in *P. lima*. PKA in *P. lima* was found to possess similar biochemical characteristics to that in mammals, including two different heterologous complexes that most likely contain different regulatory subunits. I also found evidence for the presence of PKA in *Prorocentrum micans* and *Prorocentrum minimum*, two marine dinoflagellates that do not produce okadaic acid.

The identification of PKA in Prorocentrale dinoflagellates is the first direct characterization of a protein serine/threonine kinase in marine dinoflagellates. Taken together with the characterization of protein tyrosine kinases and phosphatases in marine dinoflagellates, my work extends our knowledge of dinoflagellate biochemistry. This work also supports the hypothesis that marine dinoflagellates are true eukaryotes, since they possess signaling molecules that are homologous to the set of signaling molecules in eukaryotes, as opposed to the mechanisms of signal transduction found in prokaryotic life. Therefore, my data clearly refute the mesokaryotic hypothesis. As such, the phylogenetic position of dinoflagellates should be re-assessed based on more recent biochemical and genetic information.

Evaluation of the Dedicated Protein Tyrosine Kinase Hypothesis

More recently, a new debate regarding the origin of various forms of signal transduction within the eukaryotic organization has emerged. For many years, researchers have known that unicellular eukaryotes employ cell cycle regulatory mechanisms involving phosphotyrosine signaling that are similar to higher metazoan eukaryotes. While protein tyrosine phosphatases have been characterized in a number of unicellular organisms, no dedicated protein tyrosine kinases have been isolated within the unicellular eukaryotes. Recent sequencing of the complete genome of the budding yeast *Saccharomyces cerevisiae* revealed that, although dual-specificity protein kinases were encoded, no open reading frames possessed sequences characteristic of dedicated protein tyrosine kinases. Therefore, previous workers have hypothesized that dedicated protein tyrosine kinases evolved among metazoans to regulate and coordinate multicellular growth and development.

I have also found no evidence for dedicated protein tyrosine kinases in marine dinoflagellates, based on molecular biological techniques involving PCR amplification of protein tyrosine kinases or hybridization of a cDNA library from *P. lima*. In addition, autophosphorylating PLIK 1A was not affected by the general protein tyrosine kinase inhibitors genistein or herbimycin A nor was PLIK 1A able to phosphorylate peptides containing consensus phosphorylation sequences for known protein tyrosine kinases. Although this evidence was not reported formally in this thesis, it has ironically facilitated a more accurate picture of the nature of PLIK 1A activity in *P. lima*. Thus, it would appear that PLIK 1A is an autophosphorylating dual-specificity kinase. The work presented in this thesis not only supports the hypothesis that dinoflagellates are true eukaryotes, but also clarifies the emerging picture of the evolution of dedicated protein tyrosine kinases within the eukaryotic organization. Ironically, dinoflagellates were originally excluded from this group.

I have demonstrated that marine dinoflagellates represent a workable system for the investigation of signal transduction. My initial objective was to examine the nature of signal transduction in these organisms and determine if the production of the potent PP-1 inhibitor okadaic acid by *P. lima* influenced signal transduction in that dinoflagellate. Taken together, the evidence suggests that marine dinoflagellates possess signal transduction mechanisms homologous to those sensitive to okadaic acid in mammals. Therefore, the hypothesis that *P. lima* and dinoflagellates in general possess signal transduction mechanisms that are somehow different than those in higher eukaryotes would appear to be incorrect.

Most recently, it has been demonstrated that okadaic acid appears to be sequestered to specific subcellular locations within the dinoflagellate cell [4]. Thus, toxic marine dinoflagellates such as *P. lima* confine the powerful PP-1 inhibitor okadaic acid to locations other than those occupied by toxin-sensitive protein phosphatases. This may contribute to the apparent resistance of dinoflagellates to okadaic acid.

6.4 ■ REGULATION OF PROTEIN PHOSPHATASE-1 BY REVERSIBLE PROTEIN PHOSPHORYLATION IN LOWER EUKARYOTES

In this thesis, characterization of PKA activity in marine dinoflagellate whole cell lysates suggested that a protein serine/threonine phosphatase activity might be negatively regulated by PKA. When a 600 basepair fragment of the cDNA encoding PP-1 from *P. lima* was sequenced, it was noted that the encoded sequence possessed a classic PKA phosphorylation consensus sequence around a putative phosphoacceptor Ser-145. This sequence was present among a significant number of lower eukaryotes, while it was completely lacking among mammalian isoforms of PP-1. Therefore, I hypothesized that the phosphatase activity being regulated by PKA activity in dinoflagellates was PP-1. Since efforts have failed to identify inhibitor-1 activity in *P. lima* cell extracts, I also suggested that direct phosphorylation of PP-1 among lower eukaryotes represented a progenitor system of negative regulation of PP-1 by PKA that among higher eukaryotes involves an intermediate polypeptide in the form of either inhibitor-1 or DARPP-32.

The Phylogenetic Position of Dinoflagellate Protein Phosphatase-1

The 600 basepair fragment of the *P. lima* PP-1 cDNA also permitted direct comparison of its sequence to the comparable fragment of PP-1 from various organisms. As PP-1 is among the most conserved eukaryotic enzymes, multisequence alignment of PP-1 sequences employing computer software such as CLUSTAL [5] has been employed to generate a phylogenetic tree of the sequences. Such an analysis has produced a tree that

\corresponds to the generally accepted phylogenetic tree within the eukaryotic radiation. Employing CLUSTAL software, I performed a multisequence alignment of all the known sequences of PP-1 containing the sequence encoded by the 600 basepair fragment of *P. lima* PP-1 and generated a phylogenetic tree based on these sequences (Fig. 6.1).

Generally, this multiple sequence alignment is very similar to that for the full primary sequence of all the known PP-1s shown in Fig. 1.18. Similar sequence groupings occur, with the plants and animals forming two distinct groups and the two *T. brucei* sequences standing apart due to their disparate N-terminal inserted sequences. The *P. lima* PP-1 sequence emerges within the unicellular eukaryote group where it is grouped with the green algae. This is a logical position for *P. lima*, since it is the only other photosynthetic unicellular eukaryote that is similar to the higher plants in its PP-1 fragment sequence.

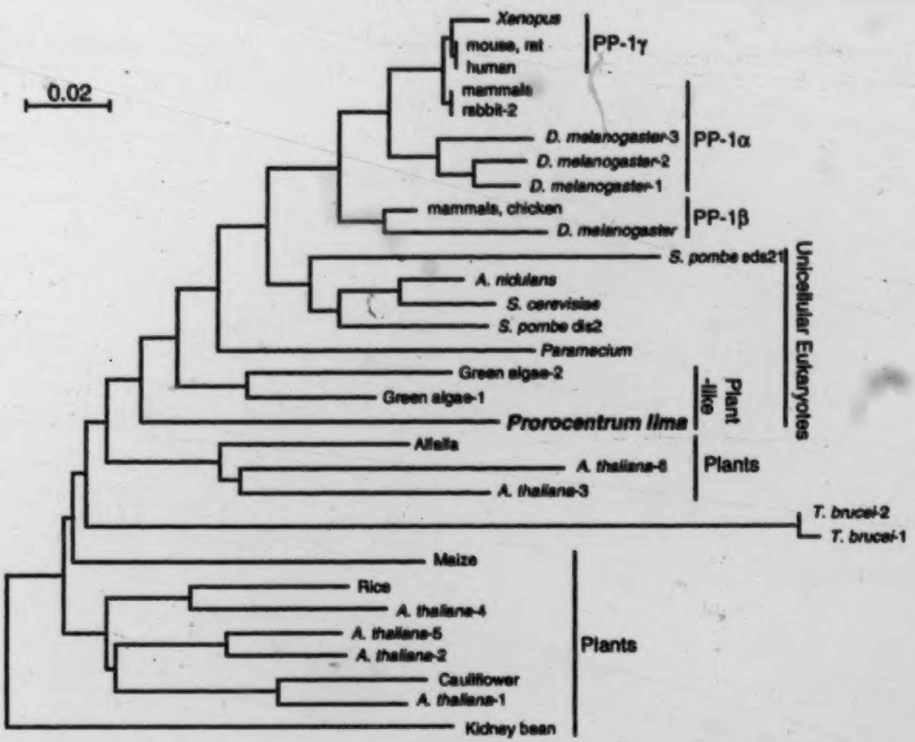

0.02
Xenopus
mouse, rat
human
mammals
rabbit-2
PP-1γ
D. melanogaster-3
D. melanogaster-2
D. melanogaster-1
PP-1α
mammals, chicken
D. melanogaster
PP-1β
S. pombe sds21
A. nidulans
S. cerevisiae
S. pombe dis2
Paramecium
Green algae-2
Green algae-1
Prorocentrum lima
Plant -like
Unicellular Eukaryotes
Alfalfa
A. thaliana-6
A. thaliana-3
Plants
T. brucei-2
T. brucei-1
Maize
Rice
A. thaliana-4
A. thaliana-5
A. thaliana-2
Cauliflower
A. thaliana-1
Kidney bean
Plants

Fig. 6.1 Phylogenetic alignment of all known sequences of PP-1 corresponding to the fragment of PP-1 from *Prorocentrum lima*. This marine dinoflagellate (in bold) is grouped with the unicellular eukaryotes in a small subgroup of sequences including the green algae.

Identification of a form of PP-1 in *S. pombe* that is Resistant to Inhibitors

Although my initial hypothesis that PP-1 from a number of lower eukaryotes might be regulated by phosphorylation of the catalytic subunit by PKA was formulated from insights derived from the marine dinoflagellate system, I chose to test this hypothesis in the fission yeast *Schizosaccharomyces pombe*. This unicellular eukaryote has been extensively characterized biochemically. The *S. pombe* genome encodes two isoforms of PP-1. One of these, referred to as sds21, contains the putative PKA phosphorylation site. The other, termed dis2, does not contain the putative PKA site. Mutant strains of *S. pombe* in which one of the PP-1 genes had been knocked-out had already been constructed and were available.

Initial characterization of PP-1 from wild type *S. pombe* cells revealed the presence of a high molecular mass phosphorylase phosphatase that was resistant to all relevant phosphatase inhibitors, thereby confounding its identity as a type-1 or 2A protein serine/threonine phosphatase. I termed this activity PP-r (protein phosphatase, resistant). Purification of sds21 from *S. pombe* in which the *dis2⁺* gene had been knocked out (Δdis2 *S. pombe*) failed to produce any free catalytic sds21. However, the phosphatase activity contained in Mono-Q fractions corresponding to PP-1 from wild type *S. pombe* was resistant to all inhibitors tested, characteristic of PP-r.

The hypothesis that PP-r contains sds21 as the catalytic subunit was tested by performing parallel PP-1 purifications employing cell lysates from Δdis2 and Δsds21 *S. pombe*. The results of this study, explained in detail in Chapter 4 of this thesis, strongly suggest that PP-r contains sds21 activity.

Previous workers chromatographically characterized both PP-1 and PP-2A from fission yeast and did not observe PP-r activity [6]. However, this work was done only at the level of DEAE anion exchange chromatography of cell lysates. My purification protocol extended this work by rechromatographing the PP-1 containing DEAE fractions on Mono-Q anion exchange chromatography. It was only at this stage of purification that PP-r activity was observed. Given the low amount of PP-r activity in wild type cells relative to free catalytic PP-1, it is perhaps not surprising that the initial characterization of PP-1 and PP-2A from *S. pombe* failed to detect PP-r activity.

The characterization of PP-r as containing sds21 activity raised some intriguing structural points. It appears that the *S. pombe* PP-1 complex is stable, regardless of the isoform of PP-1 present and that sds21 is the preferred catalytic subunit in the complex. It also appears that both isoforms of PP-1 from *S. pombe* form high molecular mass complexes. However, depending on the isoform of PP-1 in the complex, the activity reacts differently to inhibitors. This suggests that structural differences between sds21 and dis2 might explain the distinct inhibition of the two PP-1 complexes. Since PP-1 is among the most conserved eukaryotic enzymes, these structural differences must be very subtle. In this context, the phosphorylation of dis2 on Thr-320 by cdc2 kinase or the putative phosphorylation of sds21 on Ser-145 by PKA may play important roles.

The different biochemical characteristics of the two *S. pombe* PP-1 complexes suggest that the two isoforms of PP-1 in fission yeast may play different physiological roles. This hypothesis has already been suggested with the finding that dis2 activity is regulated in a cell cycle-dependent manner by cdc2 kinase phosphorylation. It has been observed that removal of the *dis2*$^+$ gene results in slower-growing fission yeast, while Δsds21 *S. pombe* grow normally. It has also been suggested that the different isoforms of PP-1 in mammals may play subtly different regulatory roles, although this idea has not gained widespread favour. My work has demonstrated that different isoforms of PP-1 from *S. pombe* are able to bind in the same complex, but that this results in different biochemical properties. Therefore, the work presented in this thesis supports the hypothesis that different isoforms of PP-1 may play subtly different regulatory roles.

Future Study of PP-r

My data represent the first definitive example of a functional difference between the same phosphatase complex containing different PP-1 isoforms. As such, it will be important in the future to determine the subunit structure of PP-r and the physiological roles in which PP-r may be involved. Thankfully, given the amenability of the *S. pombe* system to several multidisciplinary techniques, there are multiple strategies that may be employed to investigate this complex.

One such strategy is the isolation of sequenceable peptides from the PP-r complex. These peptides could then be used as the basis of an *S. pombe* cDNA library screen to determine the entire sequence of the subunits of PP-r. The nature of the interactions between *S. pombe* PP-1 and other subunits in the high molecular mass complex could be examined through mutagenesis of sds21, dis2, or any of the interacting proteins. Crystallographic determination of the structure of the PP-r complex would precisely identify the structures of sds21 that might explain the resistance of PP-r to inhibitors only when sds21 is present. Such a structural determination would also increase our understanding of inhibitor interaction with PP-1.

Since the active site of PP-1 is occupied by inhibitors, one might expect that any structure that blocks inhibition of PP-1 would also block access of substrates such as phosphorylase. However, phosphorylase is able to bind and be dephosphorylated by PP-r, while the inhibitors are apparently unable to interact with the catalytic subunit of PP-r. This suggests that a different subset of interactions occurs between PP-r and its substrate phosphorylase than PP-r and all the inhibitors tested.

Opposing this hypothesis is the fact that NaF is also ineffective as an inhibitor of PP-r. According to the hypothesis above, this would be due to the inability of NaF to interact with the catalytic subunit of PP-r. Conversely, phosphorylase, a large protein, is able to interact such that it is dephosphorylated by PP-r. Is it possible, then, that the subunits of PP-r confer phosphorylase-specificity to sds21? Recall that the association of PP-1 catalytic subunit with the glycogen binding subunit in mammalian skeletal muscle conveys increased activity toward glycogen-associated PP-1 substrates to that complex, known as PP-1G. Intriguingly, these substrates include phosphorylase, the substrate I employed to characterize PP-r. In addition, the binding of PP-1G to glycogen particles also increases their resistance to cytosolic inhibitors. Another coincidence is that the PP-1G complex from mammalian muscle is bound to glycogen particles and, as such, elutes from gel filtration at a position characteristic of very high but variable molecular mass. PP-r was also found to possess very high and variable molecular mass. Is PP-r the fission yeast homolog of PP-1G?

To test this hypothesis, it will be important to compare the substrate specificity of PP-r employing different substrates such as phosphorylated myosin light chain, which is employed to assay PP-1 activity bound to myosin-binding subunits. An alternative method would be to compare the activity and inhibitor sensitivity of PP-r in the presence and absence of compounds which chemically break down glycogen. If the biochemical characteristics of PP-r are due to its binding glycogen, we would expect to see reduced activity of PP-r toward phosphorylase and increased sensitivity to the inhibitors when glycogen is removed from the assays.

Expression and Purification of sds21 in *E. coli* and *S. pombe*

Although the characterization of PP-r activity from *S. pombe* raised new questions about the regulation of PP-1 by association with targeting subunits and re-opened the hypothesis of different physiological roles mediated by different isoforms of PP-1, my focus was on the hypothesis that PP-1 from several lower eukaryotes may be phosphorylated by PKA on Ser-145 and thereby regulated directly by the action of PKA. It was concluded that insufficient free catalytic sds21 could be purified from Δdis2 *S. pombe* to unambiguously test this hypothesis.

Therefore, a different approach to the problem was taken. The cDNA encoding sds21 was obtained and vectors constructed to express and purify recombinant sds21 in *E.*

coli. In anticipation of future experiments involving immunoprecipitation, a vector for the expression of hemagluttinin-epitope tagged sds21 in *E. coli* was also constructed. I found that the expression of the epitope-tagged sds21 caused a reduction in cell growth, although the protein that was expressed was recognized by 12CA5 monoclonal antibodies directed against the epitope tag in whole cell lysates. Given the effect of epitope-tagged sds21 expression in *E. coli*, only unmodified sds21 was expressed for purification. However, I found that, unlike human PP-1γ which is expressed in soluble form at high levels in *E. coli*, sds21 was expressed as insoluble protein.

This posed a serious technical challenge since there was no documented protocol for the purification of PP-1 from inclusion bodies in *E. coli*. However, denaturation and renaturation of insoluble PP-1α expressed in a baculovirus system had been reported by others [7]. I therefore developed my own protocol which was a convenient and rapid method permitting purification of sds21 within four days of isolating a colony of *E. coli* cells harbouring the expression vector. This protocol has the potential to advance PP-1 research because many mutants of PP-1 constructed to study structure-function relationships of the enzyme are insoluble and are therefore a challenge to purify. This straightforward purification protocol made it possible to study not only phosphorylation of sds21 by PKA, but also to test other hypotheses that required purification of PP-1 mutants.

I also overexpressed sds21 in *S. pombe*. Ideally, sds21 would be expressed in Δdis2 *S. pombe* such that the only isoform of PP-1 in these cells would be sds21. However, several attempts failed to successfully transform Δdis2 *S. pombe* with the appropriate expression vector. It is possible that since Δdis2 *S. pombe* is already cell growth-compromised, further insult to the cells through the alkali transformation protocol abolishes their ability to grow altogether. Transformation of Δsds21 *S. pombe* with the sds21-containing expression vector was possible and, in that system, sds21 was expressed at high levels in soluble form. Overexpressed sds21 was purified from *S. pombe* and possessed all of the biochemical characteristics of native free catalytic sds21.

Since the expression of epitope-tagged sds21 caused detrimental effects on cell growth in *E. coli*, it was important to determine if overexpression of the same epitope-tagged sds21 in *S. pombe* would have a similar effect. I found that overexpression of epitope-tagged sds21 in *S. pombe* had no ill effects on cell growth and that, like unmodified sds21, the epitope-tagged protein was expressed in soluble form at high levels. Therefore epitope-tagged sds21 from *S. pombe* was purified and tested for its ability to be recognized by the 12CA5 monoclonal antibodies. Epitope-tagged sds21 expressed in *S pombe* was not recognized by the antibody, unlike the same protein expressed in *E. coli*. However, it has been noted that multiple epitope tags may be required for the recognition of proteins expressed in yeast cells [8]. Since the epitope-tagged version of sds21 possessed one copy of the epitope tag, it will be important to examine whether multiple copies of the epitope tag on sds21 expressed in *S. pombe* may be antigenic for future studies investigating the interaction of sds21 with other subunits. An example of this would be the PP-r complex.

The Hypothesis of Direct Regulation of Protein Phosphatase-1 by cAMP-dependent Protein Kinase among Lower Eukaryotes

Having successfully expressed and purified recombinant sds21, I set out to test the hypothesis that it is phosphorylated by PKA. A mutant version of sds21 in which the putative phosphoacceptor Ser-145 was substituted with alanine, termed the S145A sds21 mutant, was also constructed. Both wild type and S145A sds21 were included in PKA phosphorylation reactions. Low level PKA-dependent phosphorylation of both forms of sds21 was observed. It has been noted by previous workers that low level phosphorylation of PP-1 from *S. pombe* that has been isotopically-labeled occurs *in vivo*. However, since both wild type and mutant forms of sds21 are phosphorylated, it appears that sds21 is not phosphorylated on Ser-145 by PKA.

Closer examination of the crystal structure of PP-1 revealed that, although the homologous residue in rat PP-1 is at the surface and exposed to solvent, it is located at a hairpin turn between two helices. This constrained structure is in contrast to the extended conformation of several PKA substrates, represented by the pseudosubstrate inhibitor of PKA known as PKI (protein kinase inhibitor). Comparison of the crystal structures of PKI when bound to PKA and the region of PP-1 that contains the putative phosphorylation site demonstrates that the PP-1 site possesses a structure that is not amenable to binding by PKA. This advances our knowledge of PKA-substrate interactions because very little evidence has dealt with the effect of higher order structure on the efficiency of interaction of protein kinases with phosphorylation consensus sequences. My work suggests that, for PKA, an extended conformation of the consensus sequence is required for the efficient phosphorylation of that site.

Investigation of marine dinoflagellates lead to the hypothesis that PP-1 might also be regulated by direct phosphorylation of the catalytic subunit by PKA for some isoforms of PP-1 among lower eukaryotes. I experimentally tested this hypothesis and found it to be untrue. However, the work with *S. pombe* PP-1 described in this thesis lead to some significant advances in our understanding of the regulation of PP-1 by targeting subunits. I found that complexes containing one of the two isoforms of PP-1 in *S. pombe* possess functional differences. This suggests that different isoforms of PP-1 might serve different physiological roles, based on very subtle differences in their structure. My work is the first unambiguous evidence in support of this hypothesis, which in mammalian systems has not been widely accepted. As such, my work may justify future studies of functional differences between PP-1 isoforms. In mammalian systems, there are many genes encoding three different isoforms. Thus, the *S. pombe* system, where only two genes encode two different isoforms, represents an excellent system for future studies.

6.5 ■ THE MOLECULAR MECHANISM OF INHIBITION OF PROTEIN PHOSPHATASE-1 BY MARINE TOXINS

PP-1 is a cellular receptor of several toxins derived from marine sources, including okadaic acid, microcystin-LR and nodularin. Since the inhibition of PP-1 by these toxins leads to detrimental health effects in humans, it is important to understand the mechanism of inhibition of PP-1 by the marine toxins. My work was based on the hypothesis that studying signal transduction in the marine dinoflagellate *Prorocentrum lima*, which is a primary producer of okadaic acid, would provide insight into the regulation of PP-1 by marine toxins. This work led to the study of phosphorylation of PP-1c by PKA among several lower eukaryotes as a mechanism of PP-1 regulation. My results provided evidence that interaction of different isoforms of PP-1 with the same targeting subunits may result in different physiological roles for these isozymes. In Chapter 5, I returned to the theme of the regulation of PP-1 by marine toxins and outlined an experimental strategy, based on PP-1 mutagenesis, to elucidate the molecular mechanism of inhibition of PP-1 by the toxins.

Specific amino acids of PP-1 were selected for mutagenesis based on two sources of information. One was the crystal structure of PP-1 itself. The crystal structure of PP-1 with microcystin-LR bound provided insight into the specific interactions between the phosphatase and the inhibitor. The other was molecular modeling of nodularin and okadaic acid docked into the toxin binding site of PP-1. The latter provided clues as to the possible interactions between PP-1 and those toxins.

Characterization of a Common Marine Toxin Binding Site

I found that hydrophobic interactions between the hydrophobic regions of the marine toxins and PP-1 are very important to inhibition. Mutation of Trp-206, thought to be a key contributor to hydrophobic interactions, resulted in profound increases in resistance of PP-1 to the marine toxins. This supports the hypothesis of previous workers which suggests that hydrophobic interactions might represent the driving force for the initial interaction between PP-1 and the marine toxins and contribute to the high affinity binding of the toxins. My work also suggests that Trp-206 is important for the normal activity of the enzyme, since all Trp-206 mutants possess about 150-times less specific activity than wild type enzyme. It will be important in the future to determine the K_M of different substrates and the k_{cat} of the mutant to elucidate the mechanism in which Trp-206 plays important roles.

It was also hypothesized that Tyr-134 forms a hydrogen bond with microcystin-LR and nodularin from the crystal structure and molecular models. Mutation of Tyr-134 to Phe resulted in an approximately 10-fold increase in resistance to the cyclic peptide inhibitors in support of this hypothesis. However, inhibition by okadaic acid was not changed upon the mutation of Tyr-134 to Phe, suggesting that no hydrogen bond occurs between PP-1 and okadaic acid. Mutation of Tyr-134 to alanine resulted in profound increases in marine toxin resistance of the mutant, suggesting that hydrophobic interactions are the main factor contributing to PP-1 sensitivity at this amino acid.

The hypothesis that the marine toxins bind to a common toxin binding site on PP-1 was the impetus for docking the free structures of okadaic acid and nodularin onto the crystal structure of PP-1. The results of my work support the hypothesis that the marine toxins bind to the same region of PP-1, but also suggest that specific interactions play different roles in the binding of the various marine toxins. For example, all of the marine toxins interact with Tyr-134 of PP-1, but only the cyclic peptide inhibitors do so through a hydrogen bond. Conversely, okadaic acid relies on hydrophobic interaction with Tyr-134 to a greater extent than microcystin-LR and nodularin.

Identification of a Potential Toxin Binding Site on Protein Phosphatase-2B

Another source of amino acid residues in PP-1 that might play roles in interactions with the marine toxins followed from my comparison of the crystal structures of PP-1, which is sensitive to inhibition by the toxins, and PP-2B, which is relatively resistant. My work is the first to suggest the presence of a potential toxin binding site in PP-2B that possesses conserved amino acids in positions that are important for the interaction of PP-1 with the marine toxins. As such, PP-2B could potentially bind marine toxins and thereby be sensitive to inhibition by them. I suggested that structural differences between PP-1 and PP-2B surrounding the toxin binding site is responsible for the disparate sensitivity of these two closely related phosphatases to the marine toxins.

Therefore, I compared the crystal structures of PP-1 and PP-2B and identified amino acids that might block marine toxins from entering the active site of PP-2B. I hypothesized that by substituting homologous amino acids in PP-1 with amino acids from PP-2B, I could measure increases in toxin resistance of PP-1 mutants containing amino acids that might contribute to PP-2B marine toxin resistance. In addition, these studies would also implicate amino acids in PP-1 that contribute to its sensitivity to the marine toxins.

Investigation of the L7 Loop Hypothesis

Mutants derived from the comparison of the crystal structures of PP-1 and PP-2B demonstrated that His-155, Tyr-159, Phe-160, and Cys-256 of PP-2B contribute only slightly to the increased resistance of PP-2B to the marine toxins. Therefore, some other structural feature of PP-2B is responsible for the resistance of PP-2B to the marine toxins.

My comparison study pointed to the L7 loop of PP-2B as playing a role in toxin resistance. Closer comparison of the L7 loops of PP-1 and PP-2B revealed a radically different backbone conformation between the two loops. In the case of PP-1, the conformation forms part of the toxin binding region of PP-1 that permits access of the toxins to the active site of the enzyme. In the case of PP-2B, the conformation of the L7 loop is such that the sidechains of the loop extend into the potential toxin binding region of PP-2B. I therefore hypothesized that the radically different backbone conformation of the PP-2B L7 loop is responsible for the resistance of the phosphatase to the marine toxins.

I also hypothesized that the conformation of the L7 loop of PP-2B was intrinsic to the amino acids of the loop itself. I concluded that single mutations in the L7 loop of PP-1 would not be sufficient to emulate the backbone conformation of the PP-2B L7 loop and decided that mutating all five amino acids of the L7 loop of PP-1 would have to be performed. Changing five amino acids in a single round of mutagenesis with the system I employed certainly pushed the limits of the technique, but I was able to obtain a single clone that possessed the correct mutation. I purified what was known as the PP-1 L7 Loop mutant and found that its resistance to the marine toxins was not significantly different than wild type PP-1.

This raised the question: what is the backbone conformation of the L7 loop in the PP-1 L7 Loop mutant? Since comparison of the crystal structures of PP-1 and PP-2B strongly suggested that the L7 loop of PP-2B blocks marine toxin access to the active site, I suspected that the conformation of the L7 loop in the PP-1 L7 Loop mutant corresponded to that of wild type PP-1. A corollary of this hypothesis is that, in the PP-1 L7 loop conformation, the amino acids of the PP-2B L7 loop do not confer resistance to the marine toxins.

My hypothesis raised another question: what accounts for the radically different L7 loop conformation in PP-2B if it is not intrinsic to the amino acids of the loop itself? I reasoned that some other structural feature of PP-2B that is distal to its potential toxin binding site present in PP-2B but lacking in PP-1 must interact with the L7 loop of PP-2B. The most logical candidate for such a structure would be within the extended C-terminal tail of PP-2B which is completely lacking in PP-1. I therefore examined the crystal structure of PP-2B with particular attention to any possible interactions between the L7 loop and the C-terminal tail. I discovered that His-339 of the C-terminal tail may form an interaction with Asp-313 of the L7 loop that constrains the backbone of the loop into the conformation that I believe is responsible for the resistance of PP-2B to the marine toxins.

The work described in this thesis has provided a refined picture of the structural rationale for the resistance of PP-2B to the marine toxins. Interactions between the L7 loop of PP-2B with the C-terminal tail of PP-2B result in changes in the L7 loop backbone conformation relative to the comparable loop in PP-1 such that the sidechains of the PP-2B L7 loop block the potential marine toxin binding site of PP-2B. This is the first hypothesis to satisfactorily explain PP-2B toxin resistance. As such, this hypothesis could be tested in future experiments by mutating His-339 of PP-2B such that the proposed interaction between it and Asp-313 of the L7 loop is lost. I predict that this would relax the L7 loop of PP-2B such that it takes on a backbone conformation more like that of PP-1. Characterization of the PP-1 L7 Loop mutant suggests that in the conformation of PP-1, the amino acids of the PP-2B L7 loop do not confer marine toxin resistance to the phosphatase. Therefore, simply removing the interaction between Asp-313 and His-339 of PP-2B by mutating His-155 should result in a form of PP-2B that is sensitive to inhibition by the marine toxins.

Such a form of PP-2B may represent a valuable research tool. Since okadaic acid is a cell permeable toxin, investigation of the *in vivo* roles of PP-2B could be facilitated by an okadaic acid-sensitive form of PP-2B. For example, parallel analysis of the *in vivo* phosphorylation status of proteins in cell lines that express wild type and marine toxin-sensitive PP-2B could lead to the discovery of novel substrates and physiological roles of PP-2B. In addition, by adding PP-2B to the list of major protein serine/threonine phosphatases that are sensitive to inhibition by the marine toxins, the physiological roles of PP-2C, which would be the only major protein serine/threonine phosphatase that is active in cells exposed to inhibitory concentrations of the toxins, would more easily be delineated.

My work adds to the growing body of knowledge suggesting that the marine toxins bind to the same site on PP-1 and extends our knowledge of the precise amino acids of PP-1 that are involved in the molecular mechanism of inhibition of PP-1 by these toxins. My research has also made significant contributions to our knowledge of the molecular mechanism of PP-2B resistance to the marine toxins. As such, the work presented in Chapter 5 of this thesis met the goal of furthering our understanding of the molecular mechanism of inhibition of PP-1 by the marine toxins.

Concluding Remarks

The main theme of this thesis has been the study of PP-1 regulation by reversible protein phosphorylation and marine toxins. However, as we have seen, the regulation of PP-1 does not involve the application of one regulation strategy for every physiological role, but rather involves complex interactions between several regulatory mechanisms. When studying the regulation of PP-1, one can not help but uncover new ideas about multiple modes of PP-1 regulation, since they are all interrelated at some level. As a result, although I began my work with the idea that studying signal transduction in the marine dinoflagellate *Prorocentrum lima* might provide insight into the regulation of PP-1 by okadaic acid, I discovered that isoforms of PP-1 from lower eukaryotes might also be regulated by direct phosphorylation of the PP-1 catalytic subunit. I also developed a capillary electrophoresis assay which advances our ability to detect and analyze protein phosphorylation and examined protein kinases and substrates relevant to PP-1-mediated processes. These include phosphorylation of the G subunit from PP-1G on multiple sites and hierarchical phosphorylation of glycogen synthase, a PP-1 substrate.

Investigation of the hypothesis of regulation of lower eukaryotic forms of PP-1 by direct phosphorylation of the catalytic subunit in the fission yeast *Schizosaccharomyces pombe* resulted in insights into the effect of targeting subunits on PP-1 function and provided evidence in support of the hypothesis that different isoforms of PP-1 may play different physiological roles. This would occur via interaction with different subsets of subunits resulting in different function. To facilitate this work, I developed a novel procedure for purification of insoluble recombinant PP-1.

In the end, my work came full-circle with an investigation of the molecular mechanism of PP-1 inhibition by the marine toxins. This work was made possible by my insoluble PP-1 purification protocol which permitted the purification of mutants of PP-1 that otherwise could not have been characterized. This work has raised significant new questions regarding differing molecular interactions between PP-1 and the various marine toxins. In addition, my work is the first to suggest the presence of a functional toxin binding site in PP-2B and provides a clear structural rationale explaining the resistance of PP-2B to marine toxins. As such, these studies fulfilled the aims of my thesis while presenting exciting new avenues for future research which will impact our biochemical understanding of one of the most important regulatory enzymes in eukaryotic cells.

6.6 ■ BIBLIOGRAPHY

1. Holmes, C. F. B. (1987) A new method for the selective isolation of phosphoserine-containing peptides. *FEBS Lett.* **215**: 21-24.

2. Boland, M. P., Taylor, M. F. J. R., and Holmes, C. F. B. (1993) Identification and characterisation of a type-1 protein phosphatase from the okadaic acid producing marine dinoflagellate *Prorocentrum lima*. *FEBS Lett.* **334**: 13-17.

3. Boland, M. P., and Holmes, C. F. B. (1994) Identification and characterisation of protein phosphatase-1 and -2A in marine dinoflagellates. *Adv. Prot. Phosphatases* **8**: 325-342.

4. Zhou, J., and Fritz, L. (1994) Okadaic acid antibody localizes to chloroplasts in the DSP-producing dinoflagellates *Prorocentrum lima* and *Prorocentrum maculosum*. *Phycologia* **33**: 455-461.

5. Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.* **266**: 383-402.

6. Kinoshita, N., Ohkura, H., and Yanagida, M. (1990) Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. *Cell* **63**: 405-415.

7. Berndt, N., and Cohen, P. W. T. (1990) Renaturation of protein phosphatase 1 expressed at high levels in insect cells using a baculovirus vector. *Eur. J. Biochem.* **190**: 291-297.

8. Forsburg, S. L., and Sherman, D. A. (1997) General purpose tagging vectors for fission yeast. *Gene* **191**: 191-195.

Appendix A

Phylogenetic Alignment of All Known PP-1 Sequences

Phylogenetic Alignment of PP-1c Primary Sequences

#	PP-1	Organism	SWISS-PROT Accession Number(s)
1	PP-1 α	human, rabbit, mouse, rat	P08129,P22802,P20653
2	PP-1 α-2	rabbit	P08128
3	PP-1 β	human, rabbit, mouse, rat, chicken	P37140
4	PP-1 γ	human	P36873
5	PP-1 γ	mouse, rat	P37139, Q64679, O09186, O09189
6	PP-1 γ	*Xenopus laevis*	P36874
7	PP-1-1	*Arabidopsis thaliana*	P30366
8	PP-1-2	*Arabidopsis thaliana*	P48482
9	PP-1-3	*Arabidopsis thaliana*	P48483
10	PP-1-4	*Arabidopsis thaliana*	P48484
11	PP-1-5	*Arabidopsis thaliana*	P48485
12	PP-1-6	*Arabidopsis thaliana*	P48486
13	PP-1	*Brassica oleracea* (cauliflower)	P48487
14	PP-1	*Zea mays* (maize)	P22198
15	PP-1	*Medicago varia* (alfalfa)	P48488
16	PP-1	*Oryza sativa* (rice)	P48489
17	PP-1	*Phaseolus vulgaris* (kidney bean)	P48490
18	PP-1 α-1	*Drosophila melanogaster*	P48461
19	PP-1 α-2	*Drosophila melanogaster*	P12982
20	PP-1 α-3	*Drosophila melanogaster*	Q05547
21	PP-1 β	*Drosophila melanogaster*	P48462
22	DIS2	*Saccharomyces cerevisiae*	P32598
23	dis2	*Schizosaccharomyces pombe*	P13681
24	sds21	*Schizosaccharomyces pombe*	P23880
25	PP-1-1	*Acetabularia cliftonii* (green algae)	P48480
26	PP-1-2	*Acetabularia cliftonii* (green algae)	P48481
27	PP-1	*Aspergillus nidulans*	P20654
28	PP-1-1	*Trypanosoma brucei*	P23733
29	PP-1-2	*Trypanosoma brucei*	P23734

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                      1        10                                 20                            30
1                     MSDSEKLNLD SIIGRLL------------------------EVQ GSRP----GKNVQ----------------L
2       MVTIMTTSEYLSGY ----------- --------------------------------- ------------------------------
3                     MADGE-LNVD SLITRLL------------------------EVR GCRP----GKIVQ----------------M
4                     MADLDKLNID SIIQRLL------------------------EVR GSKP----GKNVQ----------------L
5                     MADIDKLNID SIIQRLL------------------------EVR GSKP----GKNVQ----------------L

6                     MADVDKLNID SIIQRLL------------------------EVR GSKP----GKNVQ----------------L

7       MAEKPAQEQEQKRA M---EPAVLD DIIRRLV------------------------EFR NTRPGS--GKQVH----------------L
8             MAQQGQGS M---DPAALD DIIRRLL------------------------DYR NPKPG---TKQAM----------------L
9                     M-E-DSVV-D DVIKRLL------------------------GAK NGKT----TKQVQ----------------L
10        MATTTTTQQTAI ----DSAVLD DIIRRLT------------------------EVR LARP----GKQVQ----------------L
11          MAQQGQGGQQ M---DPAVLD DIIRRLL------------------------DYR NPKAG---TKQAM----------------L
12                    M-D-E-TLLD DIIRRLL------------------------ATN NGRT----VKQAQ----------------I
13    MAEKPAPEQEQEQTRA M---EPAVLD DIIRRLV------------------------EFR NTRPGS--GKQVH----------------L
14                    M---DPALLD DVIRRLL------------------------EVK NLKP----GKNAQ----------------L
15                    M-D-D-TVLD DIIKKLV------------------------SAK NGRT----TKQVH----------------L
16           MAPRRRGGG M---DPVLLD DIIPPLL------------------------EVR TARP----GKQVH----------------L
17                    M---DKAVLD DVIRRLL------------------------EG- --K-G---GKQVQ----------------L

18                    M--SDIMNID SIISRLL------------------------EVR GARP----GKNVQ----------------L
19                    M--GDVMNID SIISRLL------------------------EVR GARP----GKNVQ----------------L
20                    MA--EVLNLE SIISRLL------------------------EVR GARP----GKNVQ----------------L
21                    MGDFD-LNVD SLIQRLL------------------------EMR SCRT----GKQVQ----------------M

22                    M-DSQPVDVD NIIDRLL------------------------EVR GSKP----GQQVD----------------L
23                    MSNPD-VDLD SIIDRLL------------------------EVR GSRP----GRQVQ----------------L
24                    M-DYD---ID AIIEKLV------------------------KAR NGKP----SKQVQ----------------L
25                    M-D-E-STVD DVIERLL------------------------EVR NNRP----GKQVQ----------------L
26                    M-E-E-SAVD DVIERLL------------------------EVR NNRP----GKQVN----------------L
27                    MADQD-VDLD SIIDRLL------------------------EVR GSRP----GKQVQ----------------L
28                    M------NCD EIIKKLLLNPVHNTAATRGPAGENCESNQRTYTR ISRLAAFQSAQTQESTPKTNGTGRATTEGL
29                    M------NCR EIIRKLLLNPAHNNAATRTAQGDNGDSNQRAYTR ISRLAAFQSAQTQESTPKTNGTGRATTEGL
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                40         50         60         70         80         90        100        110        120
1       TENEIRGLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
2       ---EIRGLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
3       TEAEVRGLCI KSREIFLS-QP ILLELEAPLK ICGDIHGQYT DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
4       QENEIRGLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
5       QENEIRGLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL

6       QENEIRGLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL

7       SEGEIRQLCA VSKEIFLQ-QP NLLELEAPIK ICGDIHGQYS DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
8       NESEIRQLCI VSREIFLQ-QP NLLELEAPIK ICGDIHGQYS DLLRLFEYGG FPPTANYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
9       TEAEIKHLCS TAKQIFLT-QP NLLELEAPIK ICGDTHGQFS DLLRLFEYGG YPPAANYLFL GDYVDRGKQS VETICLLLAY KIKYKENFFL
10      SEAEIKQLCT TARDIFLQ-QP NLLELEAPIK ICGDIHGQYS DLLRLFEYGG FPPSANYLFL GDYVDRGKQS LETICLLLAY KIKYPGNFFL
11      NDSEIRQLCF VSREIFLQ-QP CLLELAAPVK ICGDIHGQYS DLLRLFEYGG FPPSANYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL
12      TETEIRQLCL ASKEVFLS-QP NLLELEAPIK ICGDVHGQFP DLLRLFEYGG YPPAANYLFL GDYVDRGKQS IETICLLLAY KVKYKFNFFL
13      SEGEIRQLCA VSKDIFLQ-QP ILLELEAPIK ICGDIHGQYS DLLRLFEYGG FPPDANYLFL GDYVDRGKQS LETICFLLAY KIKYPENFFL
14      SESEIKQLCA AAKEIFLQ-QP NLLELEAPIK ICGDVHGQYS DLLRLFDYGG YPPQANYLFL GDYVDRGKQS LETICLLLAY KVKYPENFFL
15      TEADIRQLCT SAKEIFLS-QP NLLELEAPIK ICGDVHGQFS DLLRLFEYGG YPPEANYLFL GDYVDRGKQS IETICLLLAY KIKYKENFFL
16      SESEIRQLCS VSREIFLS-QP NLLELEAPIK ICGDIHGQYS DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LETICLLLGY KIKYPENFFL
17      SESEIRQLCV HARQIFLS-QP ILLELRAPMR VCGDIHGQYQ DLLRLFEYGG YPPAANYLFL GDYVDRGKQS LETICLLLAY KIRYPDKIFL

18      SESEIRSLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYAENFFL
19      SEGEIRGLCL KSREIFLS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYSENFFL
20      SEGEIRGLCL KSREILLA-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG YPPEANYLFL GDYVDRGKQS LETICLLLAY KIKYSENFFL
21      TEAEVRGLCL KSREIFLQ-QP ILLELEAPLI ICGDIHGQYT DLLRLFEYGG FPPAANYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFL

22      EENEIRYLCS KARSIFIK-QP ILLELEAPIK ICGDIHGQYY DLLRLFEYGG FPPESNYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFI
23      SEDEIRFLCN KAREIFIS-QP ILLELEAPLK ICGDIHGQYY DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LEVICLLLAY KIKYPENFFI
24      SDAEIRYLCT TSRSIFLS-QP MLLELEAPLK ICGDIHGQYS DLLRLFEYGG YPPDANYLFL GDYVDRGKQS LEVICLLFAY KIKYPENFFL
25      TESEIRLLCL TSKEI-LSEQP NLLELEAPIK ICGDIHGQYT DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LETICLLLAF KIKYPENFFI
26      AEGEIRQLCL TAKDVFMS-QP NLLELEAPIK ICGDIHGQYT DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LETICLLLAF KVKYPKNFFL
27      LESEIRYLCT KAREIFIS-QP ILLELEAPIK ICGDIHGQYY DLLRLFEYGG FPPEANYLFL GDYVDRGKQS LETICLLLAY KIKYPENFFV
28      TEAEVRWLVM ESRALFMS-QP MLVEIAAPVR ICGDVHGQYT DLLRLFDLGG FPPDANYIFL GDYVDRGDQS LERICLLLAY KLSFPETFFL
29      TEAEVRWLVM ESRALFMS-QP MLVEIAAPVR ICGDVHGQYT DLLRLFDLGG FPPDANYIFL GDYVDRGDQS LETICLLLAY KLSFPETFFL
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                     130        140        150        160        170        180        190        200        210
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5       K-K-K----- -PNAT---RPVT PPR-GMIT-----KQAK----K

6       K-K-K----- -PNAS---RPVT PPR-GMIT-----KQAK----K

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8       R-KPRFL
9       K--------- --------R-GT PPR----------KGGG----KG
10      R-KTKFMM-S T----------- -------------KI
11      K--------- ------ILKPVD --R----------R-SRFF
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13      K-K
14      KMMG--G--S TNN-----KSGF -------------KSFRGW
15      K-KGKVGF-- GNNSS---RPGT PP-H---------KGGKN
16      R-KGKFMAS- --NKM
17      RASGSSSKM- --NF-----KK- PPK---MG-----KI

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26      K-KGKLLYGSGALAGP-GR-GTPRR
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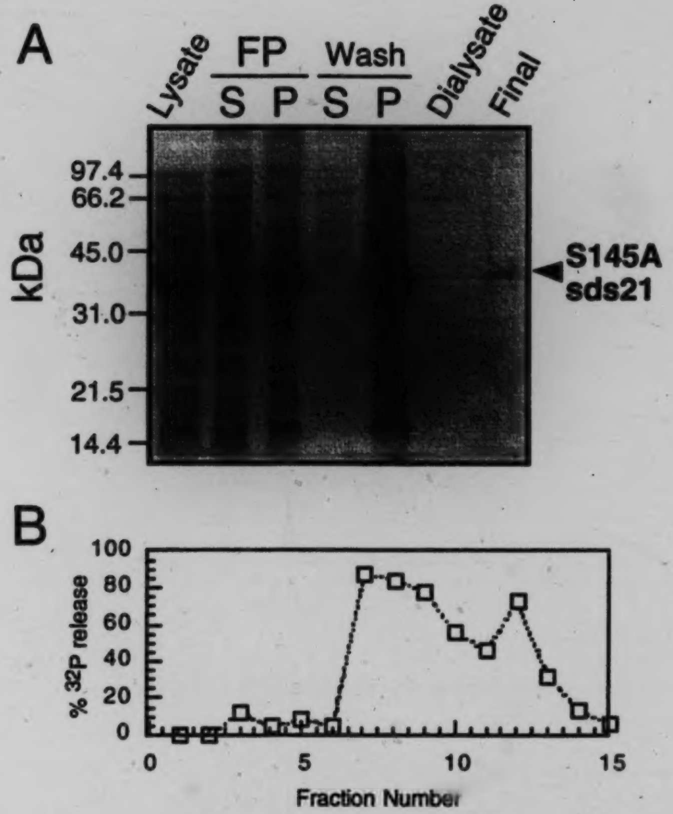
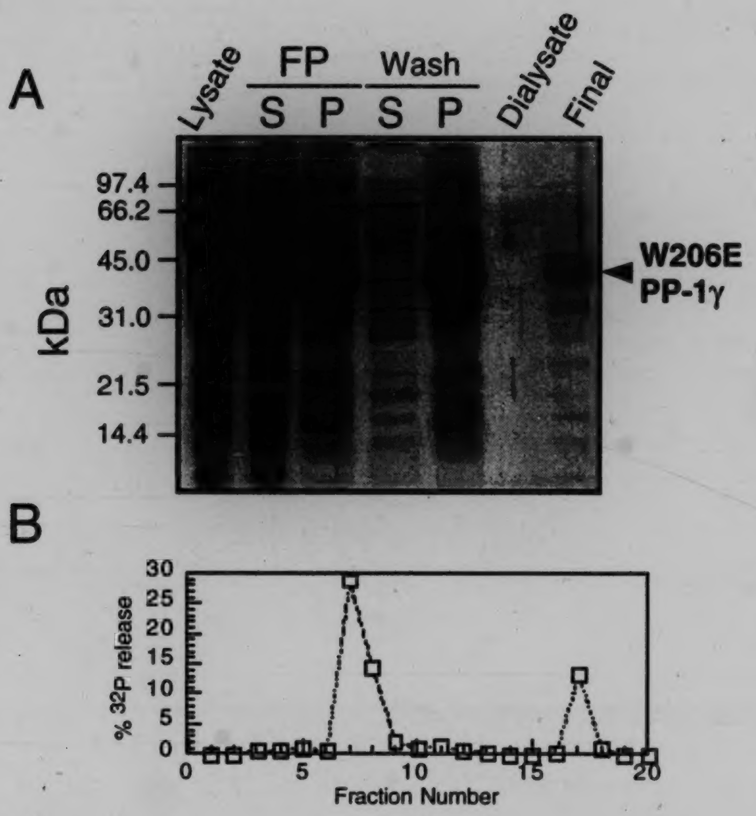
Appendix B

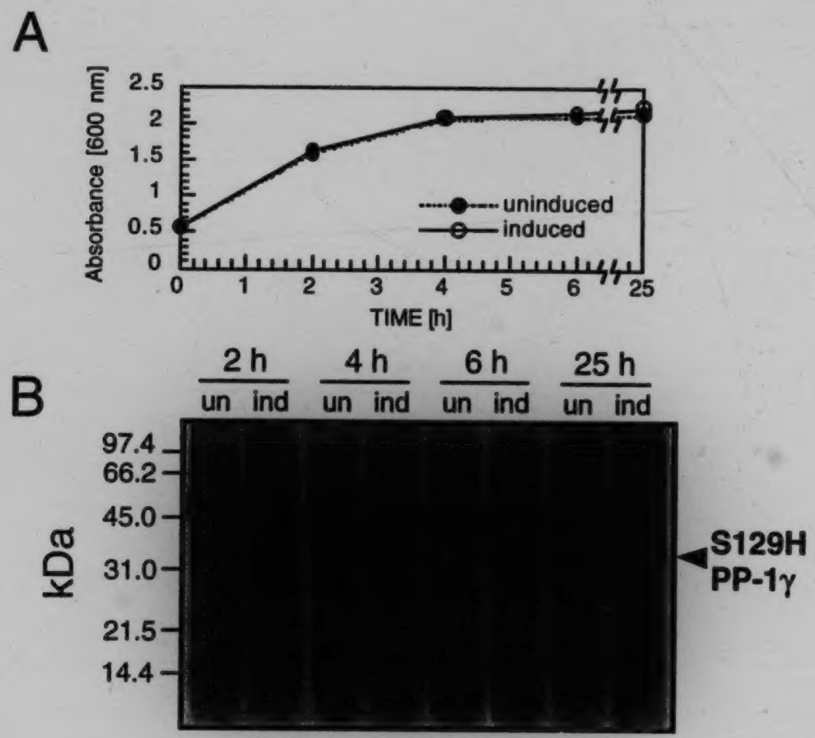
Expression and Purification of PP-1 Mutants



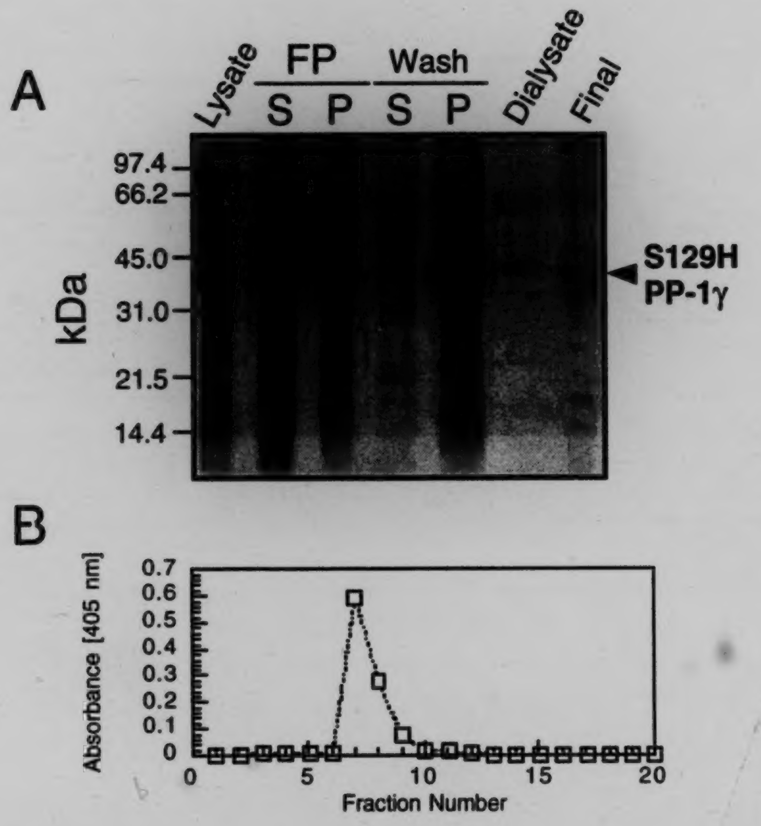
Appendix B.1 Purification of mutant S145A sds21 from insoluble inclusion bodies in *E. coli*. **A.** Progress of purification of S145A sds21. Samples run on SDS-PAGE (12%) were as described in the figure legend to Fig. 4.11. **B.** Detection of S145A sds21 activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing isotopically labelled phosphorylase as substrate, as described in Materials and Methods of Chapter 4. In this purification, 1 μL of each fraction (5 mL) was assayed. Chromatography was performed as outlined in the figure legend for Fig. 4.11.



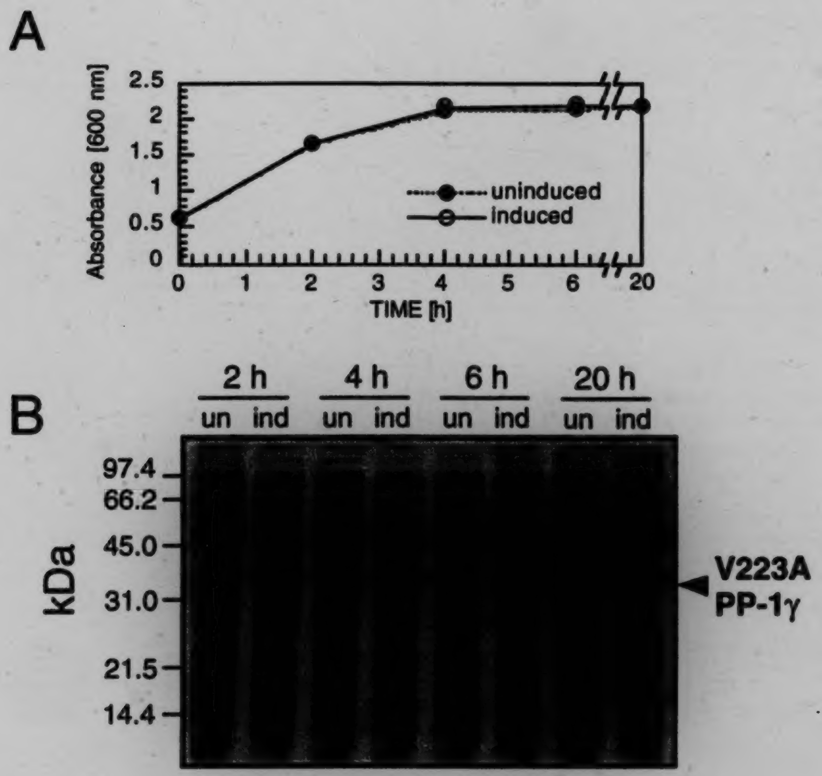
Appendix B.2 Purification of mutant W206E PP-1γ from insoluble inclusion bodies in *E. coli.* **A.** Progress of purification of W206E PP-1γ. Samples run on SDS-PAGE (12%) were as described in the figure legend to Fig. 4.11. **B.** Detection of W206E PP-1γ activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing isotopically labeled phosphorylase as substrate, as described in Materials and Methods of Chapter 4. In this purification, 1 μL of each fraction (5 mL) was assayed. Chromatography was performed as outlined in the figure legend for Fig. 4.11.



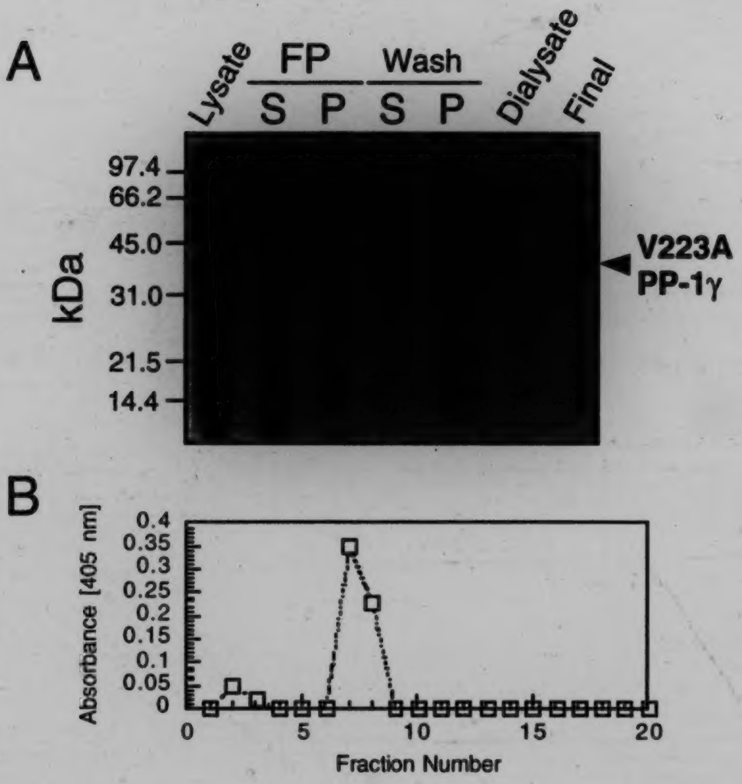
Appendix B.3 Expression of the S129H PP-1γ mutant in *E. coli*. **A.** Cell density was monitored as a function of the absorbance of a culture at 600 nm. Expression of PP-1γ in induced cultures had little effect on cellular growth. **B.** Induction of PP-1γ expression in *E. coli* over time. Samples of parallel *E. coli* cultures, one uninduced (un) and one induced (ind), were taken at time points indicated across the top and whole cell lysates analyzed by SDS-PAGE (12%). Visualization of the proteins by Coomassie Blue staining revealed increasing amounts of S129H PP-1γ mutant in cells that had been induced.



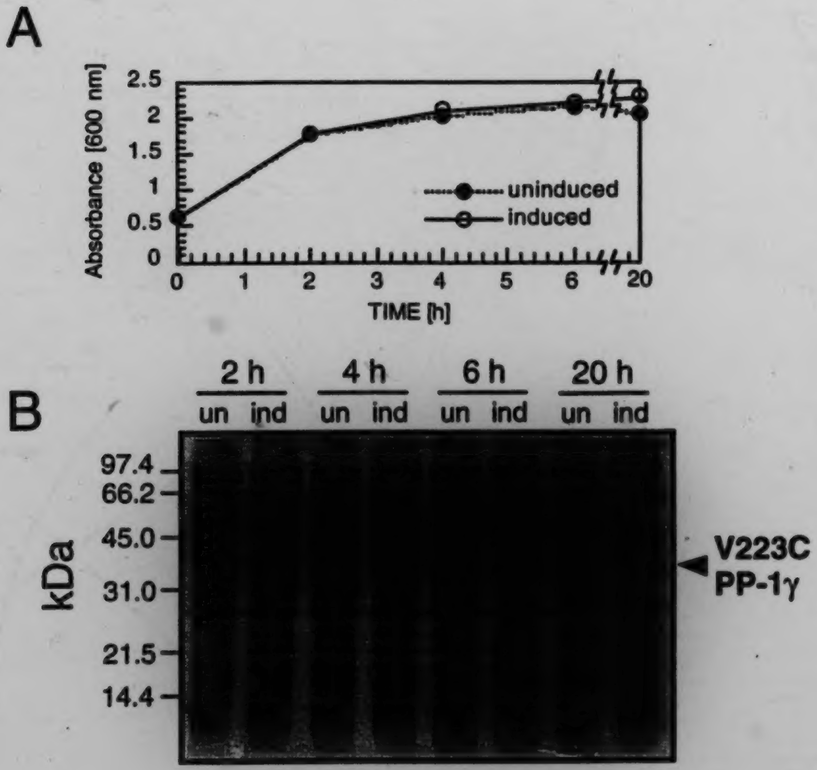
Appendix B.4 Purification of mutant S129H PP-1γ from insoluble inclusion bodies in *E. coli*. **A.** Progress of purification of S129H PP-1γ. Samples run on SDS-PAGE (12%) were as described in the figure legend to Fig. 4.11. **B.** Detection of S129H PP-1γ activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing *p*NPP as substrate, as described in Materials and Methods of Chapter 5. In this purification, 1 μL of each fraction (5 mL) was assayed. Chromatography was performed as outlined in the figure legend for Fig. 4.11.



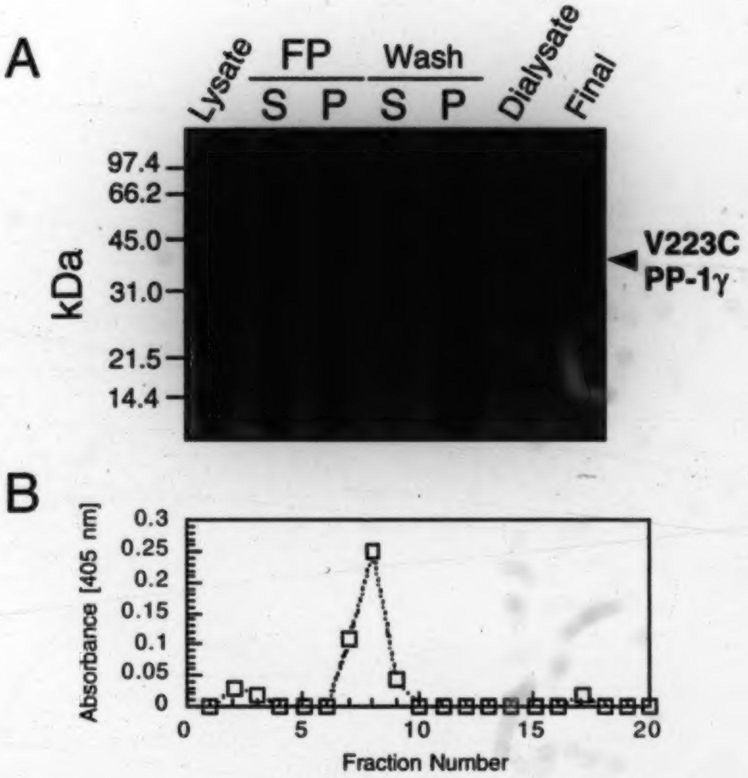
Appendix B.5 Expression of the V223A PP-1γ mutant in *E. coli*. **A.** Cell density was monitored as a function of the absorbance of a culture at 600 nm. Expression of PP-1γ in induced cultures had little effect on cellular growth. **B.** Induction of PP-1γ expression in *E. coli* over time. Samples of parallel *E. coli* cultures, one uninduced (un) and one induced (ind), were taken at time points indicated across the top and whole cell lysates analyzed by SDS-PAGE (12%). Visualization of the proteins by Coomassie Blue staining revealed increasing amounts of V223A PP-1γ mutant in cells that had been induced.



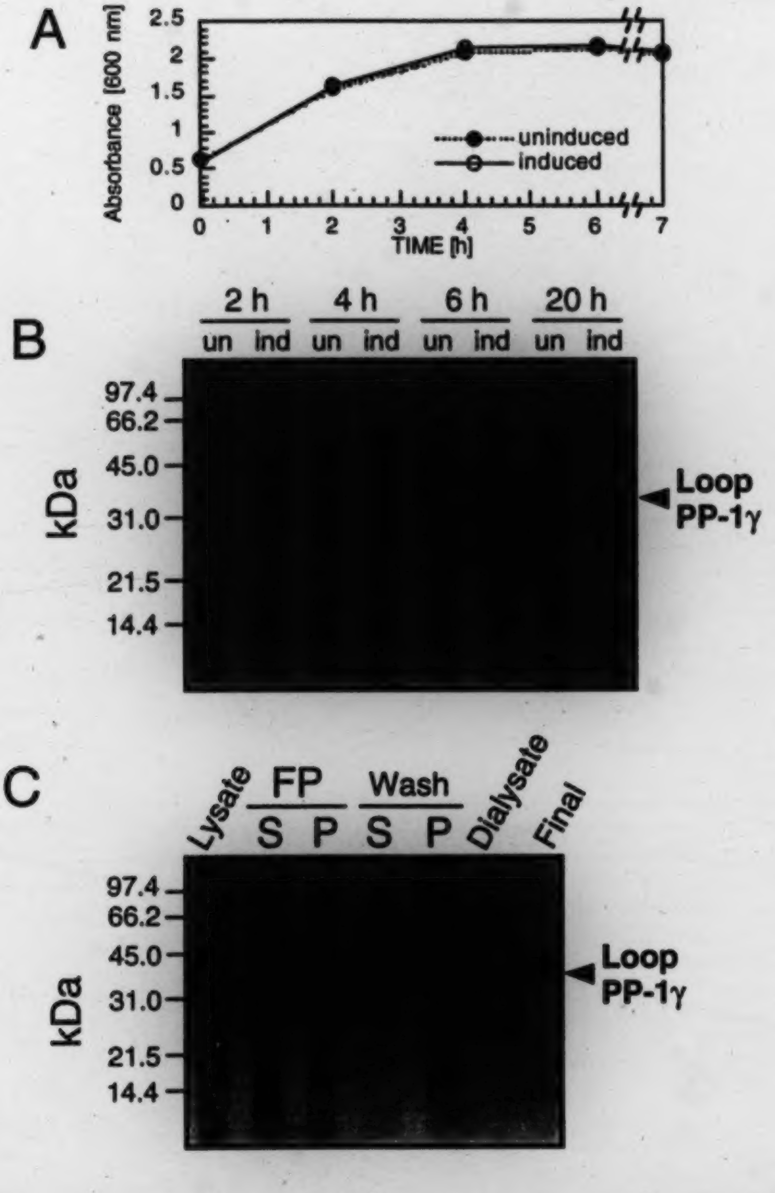
Appendix B.6 Purification of mutant V223A PP-1γ from insoluble inclusion bodies in *E. coli*. **A.** Progress of purification of V223A PP-1γ. Samples run on SDS-PAGE (12%) were as described in the figure legend to Fig. 4.11. **B.** Detection of V223A PP-1γ activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing *p*NPP as substrate, as described in Materials and Methods of Chapter 5. In this purification, 1 μL of each fraction (5 mL) was assayed. Chromatography was performed as outlined in the figure legend for Fig. 4.11.



Appendix B.7 Expression of the V223C PP-1γ mutant in *E. coli*. A. Cell density was monitored as a function of the absorbance of a culture at 600 nm. Expression of PP-1γ in induced cultures had little effect on cellular growth. B. Induction of PP-1γ expression in *E. coli* over time. Samples of parallel *E. coli* cultures, one uninduced (un) and one induced (ind), were taken at time points indicated across the top and whole cell lysates analyzed by SDS-PAGE (12%). Visualization of the proteins by Coomassie Blue staining revealed increasing amounts of V223C PP-1γ mutant in cells that had been induced. BL-21 *E. coli* were employed as the host in this case.



Appendix B.8 Purification of mutant V223C PP-1γ from insoluble inclusion bodies in *E. coli*. A. Progress of purification of V223C PP-1γ. Samples run on SDS-PAGE (12%) were as described in the figure legend to Fig. 4.11. B. Detection of V223C PP-1γ activity in Heparin-Sepharose fractions of the dialyzed renaturation reaction employing *p*NPP as substrate, as described in Materials and Methods of Chapter 5. In this purification, 1 μL of each fraction (5 mL) was assayed. Chromatography was performed as outlined in the figure legend for Fig. 4.11.



Appendix B.9 Expression and purification of the L7 Loop PP-1γ mutant from insoluble inclusion bodies in *E. coli*. **A.** Cell density was monitored as a function of the absorbance of a culture at 600 nm. Expression of PP-1γ in induced cultures had little effect on cellular growth. **B.** Induction of PP-1γ expression in *E. coli* over time. Samples of parallel *E. coli* cultures, one uninduced (un) and one induced (ind), were taken at time points indicated across the top and whole cell lysates analyzed by SDS-PAGE (12%). Visualization of the proteins by Coomassie Blue staining revealed increasing amounts of L7 Loop PP-1γ mutant in cells that had been induced. **C.** Progress of purification of L7 Loop PP-1γ mutant. Samples run on SDS-PAGE (12%) were as described in the figure legend to Fig. 4.11.

Appendix C

PPIP46 Overlay Procedure for the Detection of PP-1

C.1 ■ INTRODUCTION

A constant challenge associated with my work was the unambiguous identification of protein phosphatase-1 (PP-1) activity. More specifically, I was interested in being able to identify PP-1 by immunological means following the stimulation of cAMP-dependent protein kinase (PKA) in *S. pombe* whole cell extracts and *in vivo* to investigate my hypothesis that the sds21 isoform of PP-1 in *S. pombe* was regulated by PKA phosphorylation, as outlined in Chapter 4 of this thesis.

Presently, antibodies have been raised against C-terminal peptides from various human PP-1 isoforms, but no antibody has been produced that is capable of recognizing all the isoforms of PP-1 in any organism without cross-reacting with PP-2A. This is due to the high degree of sequence identity between these two enzymes. Previous workers claimed to have raised antibodies that specifically recognized both isoforms of *S. pombe* PP-1, in addition to antibodies that were specific for each of the two *S. pombe* PP-1 isoforms [1]. The availability of these antibodies was an added incentive to employ the *S. pombe* system for the study of the regulation of PP-1 by reversible protein phosphorylation. However, I found that all of these antibodies cross-reacted with *S. pombe* PP-2A and were therefore not useful for the characterization of PKA phosphorylation of sds21 in *S. pombe* lysates and *in vivo*.

One hypothesis that had been discussed in the laboratory was the possibility of employing the active peptide fragment of inhibitor-1, known as PPIP46, in a manner similar to that of an antibody. Inhibitor-1 was an excellent candidate because it specifically binds to all isoforms of PP-1 and does not interact whatsoever with PP-2A, thereby fulfilling the PP-1-specificity requirement. In addition, to be an active inhibitor of PP-1, PPIP46 must be phosphorylated by PKA. Thus, it was hypothesized that PPIP46 could be isotopically-labeled with ^{32}P and used to detect PP-1 in a manner similar to that of Western immunoblotting.

The experiments outlined herein demonstrate that PP-1 can be specifically detected employing isotopically-labeled PPIP46. In addition, I also demonstrate that the ability of PPIP46 to bind PP-1 is not significantly altered by prior acetone precipitation of the protein.

C.2 ■ MATERIALS AND METHODS

Materials

PPIP46 peptide was prepared as outlined in Chapter 2. Recombinant PP-1β was purified by Ms. H. A. Luu. Native rabbit PP-2A catalytic subunit from rabbit skeletal muscle was prepared by Dr. M. Craig. Isotopically-labeled PPIP46 peptide was prepared as outlined in [2].

PPIP46 Overlay Procedure

Proteins were run on SDS-PAGE (12%) and transferred to nitrocellulose with transfer buffer containing no SDS. The membrane was then blocked overnight at 4 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 (v/v), 0.1% β-mercaptoethanol, 5% non-fat skim milk (w/v) and 2% BSA (w/v). After rocking gently for 30 min, the membrane was washed three times, 15 min each time, in 100 mL TBSTB (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (v/v), and 0.1% β-mercaptoethanol) at 30 °C employing a hybridization oven. The membrane was then incubated at 30 °C in 50 mL TBSTB containing isotopically-labeled PPIP46. The membrane was then washed three times once again in 100 mL TBSTB at 30 °C for 15 min each wash, wrapped in plastic wrap and exposed to a phosphorimager plate or film to produce an autoradiogram.

Acetone Precipitation

To 100 μL of protein solution was mixed 400 μL of cold acetone (-20 °C). This solution was then incubated overnight at -20 °C. The solution was centrifuged in a desktop centrifuge for 5 min at 16000 *g* (r_{av}=7.3 cm) in a cold room (approximately 8 °C) and the resulting pellet washed with 500 μL cold acetone (-20 °C). This washing mixture was incubated at -20 °C for 10 min prior to centrifugation for 5 min in a cold room desktop centrifuge as above. The resulting pellet was air dried and solubilized in SDS-PAGE loading buffer prior to incubation at 85 °C for 8 min and running on SDS-PAGE (12%). Proteins treated in this manner were then subjected to the PPIP46 overlay procedure outlined in the previous section.

C.3 ■ RESULTS AND DISCUSSION

Aliquots of recombinant PP-1β and native PP-2A were run on SDS-PAGE, transferred to nitrocellulose in the absence of SDS and then subjected to the PPIP46 overlay procedure outlined in the Materials and Methods section (Fig. C.1A). PPIP46 was able to specifically recognize recombinant PP-1β. This result is remarkable for two reasons. First, it suggests that PP-1 is capable of refolding following the removal of SDS when bound to nitrocellulose such that it possesses structures necessary for interaction with PPIP46. Second, it also demonstrates that the PPIP46 overlay procedure was able to detect recombinant PP-1, which is typically 100- to 500-fold less sensitive to inhibition by inhibitor-1 than native enzyme [3], presumably due to subtle structural differences.

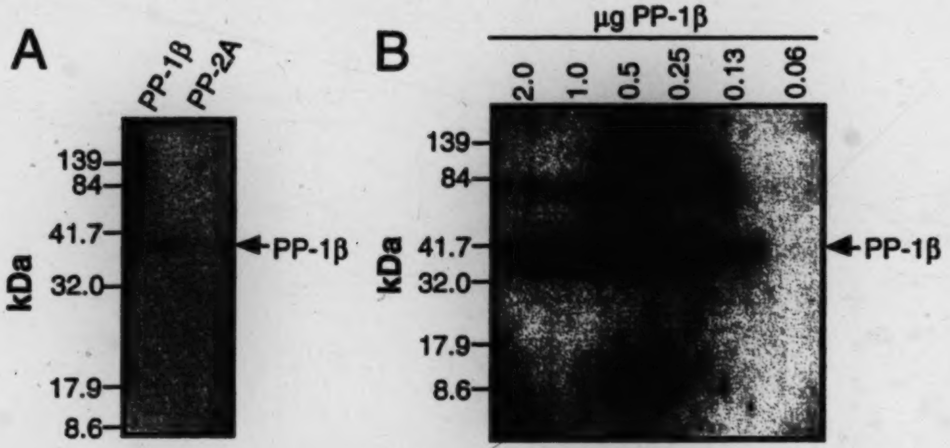


Fig. C.1 **A.** Detection of PP-1 employing the PPIP46 overlay procedure outlined in the Methods and Materials section. PP-1 was detected whereas PP-2A was not bound by PPIP46. **B.** Detection of varying concentrations of PP-1 employing the PPIP46 overlay procedure. PP-1 was clearly detected when amounts greater than 0.13 μg were run on SDS-PAGE.

It was important to determine whether the PPIP46 overlay procedure could detect varying amounts of PP-1. Therefore, I ran different concentrations of recombinant PP-1β on SDS-PAGE and followed the overlay procedure outlined above (Fig. C.1B). This result demonstrated that the PPIP46 overlay procedure was capable of detecting very small amounts of enzyme, with a threshold level of approximately 0.13 μg.

I wished to test the PPIP46 overlay procedure employing whole cell extracts to determine if PP-1 could be detected in complex mixtures. However, I faced the problem of maximizing the amount of PP-1 that could be loaded onto SDS-PAGE. Therefore, it was decided that precipitating protein from a whole cell extract with cold acetone would serve to both concentrate the protein from a large volume into a smaller volume that could be easily loaded onto SDS-PAGE and remove nucleic acids and other compounds from the sample. However, the effect of prior precipitation with acetone on the ability of PP-1 to bind PPIP46 was unknown. Therefore, a control precipitation was performed employing purified recombinant PP-1β in which the ability of the PPIP46 overlay procedure to detect precipitated PP-1 and untreated PP-1 was compared (Fig. C.2). PPIP46 was able to bind to acetone-precipitated PP-1 that had also been denatured through SDS-PAGE and renatured during transfer to nitrocellulose, although some loss in reactivity was observed.

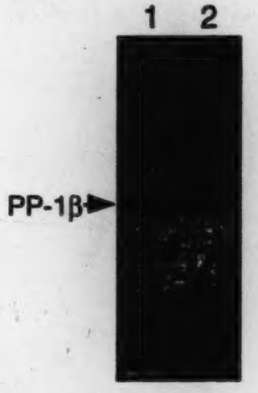


Fig. C.2 Detection of purified PP-1 (lane 1) and the identical amount of purified PP-1 that had been precipitated with acetone prior to running on SDS-PAGE (12%, lane 2). In both samples, PP-1 is clearly detected, although some loss in sensitivity is observed when PP-1 is precipitated with acetone.

The ability of the PPIP46 overlay procedure to detect PP-1 in whole cell lysates was tested. In this case, very high levels of background radiation were retained on the membrane. Therefore, the PPIP46 overlay might be more useful for the detection of PP-1 during purification in fractions that possess less contaminating compounds.

These simple experiments are striking because they suggest that PP-1 can be precipitated, denatured and refolded into a conformation that is still capable of binding PPIP46. This is remarkable when we consider that even slight structural changes in the enzyme lead to dramatic losses in PPIP46 sensitivity. In addition, these experiments were remarkable to my colleagues in the laboratory because in our experience PP-1 activity is very susceptible to inactivation during normal handling. As such, it is commonly believed that PP-1 is a "fragile" enzyme. This procedure was developed prior to the formulation of my purification protocol for insoluble forms of PP-1 which involves denaturation and renaturation. In hindsight, it is perhaps not surprising that PP-1 is able to withstand the insults of denaturation and renaturation by the addition and removal of SDS.

It is also interesting to note that this work was performed with recombinant PP-1. Given the lower sensitivity of recombinant PP-1 to inhibition by inhibitor-1, I hypothesize that even lower concentrations of native PP-1 might be detected by my method. In addition, I note in hindsight that during transfer of protein from SDS-PAGE to nitrocellulose and in the subsequent procedures, when I believe renaturation of PP-1 was occurring in the absence of SDS, no manganese was present in the buffers. During the renaturation of PP-1 in the insoluble PP-1 purification protocol, the presence of manganese is absolutely necessary for the correct refolding of PP-1 [4]. Presumably, manganese is bound in the active site of denatured recombinant PP-1 during refolding such that it adopts the proper conformation for activity. A further modification of the PPIP46 overlay procedure might be to include manganese in all steps after running protein on SDS-PAGE to maximize the amount of correct PP-1 renaturation such that maximal PPIP46 binding occurs. Clearly, this might increase the sensitivity of the PPIP46 overlay procedure.

Detection of PP-1 in acetone precipitates of protein in whole cell lysates employing the PPIP46 overlay procedure was not tested. I found that the amount of enzyme in samples that I was not able to unambiguously identify as PP-1 or PP-2A employing dose response curves with microcystin-LR and okadaic acid was too small to be detected by my PPIP46 method and therefore did not pursue further development of the procedure. However, this simple method is the only procedure to my knowledge that can definitively identify PP-1 in a manner similar to that of Western immunoblotting. This kind of characterization could be important since PP-1 is often in complexes with inhibitor proteins *in vivo* and, as such, is not detected employing activity assays on which the identification of phosphatase activity employing marine toxin dose response curves relies. In addition, since the specific interaction between PPIP46 and PP-1 is the basis of the overlay procedure, this protocol will be useful for the examination of the molecular mechanism of inhibitor-1 binding to PP-1.

C.4 ■ BIBLIOGRAPHY

1. Yamano, H., Ishii, K., and Yanagida, M. (1994) Phosphorylation of dis2 protein phosphatase at the C-terminal cdc2 consensus sequence and its potential role in cell cycle regulation. *EMBO J.* **13:** 5310-5318.

2. Rasmussen, C., and Garen, C. (1993) Activation of calmodulin-dependent enzymes can be selectively inhibited by histone H1. *J. Biol. Chem.* **268:** 23788-23791.

3. Alessi, D. R., Street, A. J., Cohen, P., and Cohen, P. T. W. (1993) Inhibitor-2 functions like a chaperone to fold three isoforms of mammalian protein phosphatase-1 into a conformation with the specificity and regulatory properties of the native enzyme. *Eur. J. Biochem.* **213:** 1055-1066.

4. Berndt, N., and Cohen, P. W. T. (1990) Renaturation of protein phosphatase 1 expressed at high levels in insect cells using a baculovirus vector. *Eur. J. Biochem.* **190:** 291-297.